

**UNITED STATES INTERNATIONAL TRADE COMMISSION
WASHINGTON, D.C.**

**Before the Honorable Clark S. Cheney
Chief Administrative Law Judge**

In the Matter of

**CERTAIN DRUG PRODUCTS
CONTAINING C-TYPE NATRIURETIC
PEPTIDE VARIANTS, AND
COMPONENTS THEREOF**

Inv. No. 337-TA-1447

RESPONDENTS' REPLY CLAIM CONSTRUCTION BRIEF

TABLE OF CONTENTS

I.	INTRODUCTION	1
II.	PERSON OF ORDINARY SKILL IN THE ART	1
III.	DISPUTED CLAIM CONSTRUCTIONS	1
A.	“CNP variant” (claims 15–22, 31–49, 54)	1
B.	“hydrolysis” (claims 15–17 and 31–39)	3
C.	“coupled to the CNP variant through a hydrolysable linkage” (claims 15–20, 31–48)	9
D.	“formulation” (claims 18–20, 40–48)	11
E.	“sustained release CNP variant formulation” (claims 18–20, 40–48)	11
F.	“macromolecule” (claims 15–17, 31–39)	12
G.	“synthetic polymeric group” (claims 15–20, 31–48)	13
H.	“contacting the/a cell” (claims 21, 22, 49, and 54)	13
IV.	CONCLUSION	15

TABLE OF AUTHORITIES

Cases

<i>Abbott Lab'ys v. Sandoz, Inc.</i> , 529 F.Supp.2d 893 (N.D. Ill. 2007)	2
<i>Deere & Co. v. Bush Hog, LLC</i> , 703 F.3d 1349 (Fed. Cir. 2012).....	15
<i>In re Entresto</i> , 125 F.4th 1090 (Fed. Cir. 2025)	6 , 10
<i>Enzo Biochem, Inc. v. Applera Corp.</i> , 599 F.3d 1325 (Fed. Cir. 2010).....	14
<i>Fundamental Innovation Sys. Int'l LLC v. Anker Innovations Ltd.</i> , No. CV 21-339-RGA, 2025 WL 459916 (D. Del. Feb. 11, 2025)	6, 10
<i>Kopykake Enters., Inc. v. Lucks Co.</i> , 264 F.3d 1377 (Fed. Cir. 2001).....	5, 6, 10
<i>NeoMagic Corp. v. Trident Microsystems, Inc.</i> , 287 F.3d 1062 (Fed. Cir. 2002).....	9, 10
<i>Phillips v. AWH Corp.</i> , 415 F.3d 1303 (Fed. Cir. 2005) (en banc).....	3, 4, 5, 6, 15
<i>SkyHawke Techs., LLC v. Deca Int'l Corp.</i> , 828 F.3d 1373 (Fed. Cir. 2016).....	12
<i>SRI Int'l v. Matsushita Elec. Corp. of Am.</i> , 775 F.2d 1107 (Fed. Cir. 1985) (en banc)	9
<i>Vita-Mix Corp. v. Basic Holding, Inc.</i> , 581 F.3d 1317 (Fed. Cir. 2009).....	9, 10
<i>Vitronics Corp. v. Conceptiontronic Inc.</i> , 90 F.3d 1576 (Fed. Cir. 1996).....	3
<i>Warner Chilcott Co., LLC v. Zydus Pharms. Inc.</i> , C.A. No. 11-1105-RGA, 2013 WL 1729383 (D. Del. Apr. 22, 2013)	1, 2
<i>Wilson Sporting Goods Co. v. Hillerich & Bradsby Co.</i> , 442 F.3d 1322 (Fed. Cir. 2006).....	9

TABLE OF EXHIBITS

Ex. No	Description
1	U.S. Patent No. RE48,267 E (the “RE’267 patent”)
2	Curriculum Vitae – Jonathan L. Sessler
3	Carey, F., <i>et al.</i> , “Chapter 4, Study and Description of Organic Reaction Mechanisms,” <i>Advanced Organic Chemistry</i> , 3 rd Edition, Vol. A (Plenum Press, New York 1992) (“Carey Ch. 4”)
4	Carey, F., <i>et al.</i> , “Chapter 8, Reactions of Carbonyl Compounds,” <i>Advanced Organic Chemistry</i> , 3 rd Edition, Vol. B (Plenum Press, New York 1992) (“Carey Ch. 8”)
5	Veronese, F., <i>et al.</i> , “The Impact of PEGylation on Biological Therapies,” 22 <i>Biodrugs</i> 320, 323, 326 (2008) (“Veronese”)
6	<i>The Facts On File Dictionary Of Organic Chemistry</i> , Ed. Daintith (2004) 6 (“Facts on File Dictionary”)
7	<i>Glossary of Terms Used in Physical Organic Chemistry</i> (IUPAC Recommendations 1994) International Union of Pure and Applied Chemistry, Organic Chemistry Division, Commission on Physical Organic Chemistry, Hydrolysis: “Solvolysis by water” (1123); Solvolysis (“IUPAC Glossary”)
8	<i>Hawley’s Condensed Chemical Dictionary</i> , 15th Edition, Ed. Lewis (2007) (“Hawley’s”)
9	Huang, W., <i>et al.</i> , “Maleimide-Thiol Adducts Stabilized Through Stretching,” 11 <i>Nat. Chem.</i> 311, Figure 1a (2019) (“Huang”)
10	Wang, Z., <i>et al.</i> , “Hot Water-Promoted Ring-Opening of Epoxides and Aziridines by Water and Other Nucleophiles,” 73 <i>J. Org. Chem.</i> 2270 (2008) (“Wang”)
11	Yuzawa, H., <i>et al.</i> , “Anti-Markovnikov Hydration of Alkenes Over Platinum-Loaded Titanium Oxide Photocatalyst,” 3 <i>Catal. Sci. Technol.</i> 1743 (2013) (“Yuzawa”)
12	NOF Corporation Catalogue Ver. 8, (“NOF Catalogue”)
13	Meyer, M., <i>et al.</i> , “56: Natriuretic Peptides,” <i>Acute Heart Failure</i> (Springer London (2008) (“Meyer”)
14	Waschek, J. A., “Developmental actions of natriuretic peptides in the brain and skeleton,” 61 <i>Cellular and Molecular Life Sciences</i> , 2332 (2004) (“Waschek”)
15	Itoh, H., <i>et al.</i> , “Chapter 7, Cellular and Molecular Aspects of C-Type Natriuretic Peptide (CNP),” <i>Natriuretic Peptides In Health and Disease</i> (Humana Press 1997) (“Itoh”)
16	Furuya, M., <i>et al.</i> , “Structural Requirements of C-Type Natriuretic Peptide for Elevation of Cyclic GMP In Cultured Vascular Smooth Muscle Cells,” <i>Biochemical and Biophysical Research Communications</i> , 183(3) (1992) (“Furuya 1992”)
17	Potter, L., <i>et al.</i> , “Natriuretic Peptides, Their Receptors, and Cyclic Guanosine Monophosphate-Dependent Signaling Functions,” <i>Endocrine Reviews</i> , 27(1) (2006) (“Potter 2006”)

Ex. No	Description
18	July 10, 2019 Remarks (Excerpt of File History of U.S. Patent No. RE48,267)
19	July 3, 2019, Applicant-Initiated Interview Summary (Excerpt of File History of U.S. Patent No. RE48,267)
20	November 26, 2019, Nonfinal Rejection (Excerpt of File History of U.S. Patent No. RE48,267)
21	Yasoda, A., <i>et al.</i> , “Overexpression of CNP in chondrocytes rescues achondroplasia through a MAPK-dependent pathway,” <i>Nature Medicine</i> , Fig. 6 (2004) (“Yasoda”)
22	July 11, 2017, Preliminary Amendment (Excerpt of File History of U.S. Patent No. RE48,267)
23	May 10, 2019, Office Action (Excerpt of File History of U.S. Patent No. RE48,267)
24	UPC Notice with BM Infringement Action
25	U.S. Patent No. 8,598,121 (the ’121 patent)
26	Cambridge Dictionary of American English, 179 (2d ed. 2008)
27	Concise Oxford English Dictionary, 307 (11th ed. 2008)
28	O’Connor, C. “Acidic and Basic Amide Hydrolysis,” 4 <i>Q. Rev. Chem. Soc.</i> , 553 (1970) (“O’Connor”)
29	Devault, A., <i>et al.</i> , “Expression of Neutral Endopeptidase (Enkephalinase) in Heterologous COS-1 Cells, Characterization of the Recombinant Enzyme and Evidence for Glutamic Acid Residue at the Active Site” <i>The Journal of Biological Chemistry</i> , 263(8): 4033 (1988) (“Devault”)
30	Oefner C., <i>et al.</i> , “Structure of Human Neutral Endopeptidase (Neprilysin) Complexed with Phosphoramidon” <i>J. Mol. Biol.</i> 296: 341 (2000) (“Oefner”)
31	Deposition Transcript of Joshua Bliesath (October 24, 2025)

I. INTRODUCTION

Respondents' proposed constructions bring the claims in line with the specification and the scope of what the patentee clearly identified as the invention. BioMarin's constructions are generally overbroad and attempt to read limitations out of the claims. They should be rejected.

II. PERSON OF ORDINARY SKILL IN THE ART

The parties dispute the requirements of a POSA. It does not appear, however, that the POSA's experience is dispositive of any claim construction dispute. Thus, it is unnecessary to resolve the dispute at this time. Regardless, BioMarin contests Respondents' definition only "to the extent they presuppose the POSA would be focused on 'developing PEGylated CNP variants' or 'therapeutic CNP,' as the pertinent field is broader." BioMarin's Opening Brief ("BM Br.") at 7 n.5. As explained in Respondents' opening brief, the role of CNP in promoting bone growth was known in the prior art; the problem to be solved was the short half-life in the body of natriuretic peptides. Respondents do not require the pertinent field to be limited to therapeutic CNP, but note that BioMarin's competing "treatment of bone-related disorders" cannot be the pertinent field. "Bone-related disorders" would include fractures, osteoporosis, and bone cancer. A POSA in the field of RE'267 works in drug design and development for delivering therapeutic peptides.

III. DISPUTED CLAIM CONSTRUCTIONS

A. "CNP variant" (claims 15–22, 31–49, 54)

BioMarin argues that, because the Markush group lists a set of CNP variants, the term "CNP variant" itself needs no construction. BM Br. at 23. That is legally erroneous.

The mere fact that a Markush group lists species for a recited genus does not transform the genus's name into a nonce word. *See, e.g., Warner Chilcott Co., LLC v. Zydus Pharms. Inc.*, C.A. No. 11-1105-RGA, 2013 WL 1729383, at *6, 8 (D. Del. Apr. 22, 2013) (construing multiple

Markush groups, including the genus “enteric polymer,” which the Court found to impose functional limitations on the polymer species listed in the Markush group). Courts often construe genus names to confer functional or structural limitations beyond merely containing the listed species. *See, e.g., Abbott Lab’ys v. Sandoz, Inc.*, 529 F.Supp.2d 893, 908-09 (N.D. Ill. 2007) (construing “pharmaceutically acceptable polymer” to require the ability to extend release of a drug while maintaining certain pharmacokinetic limitations, where the recited pharmaceutically acceptable polymer was selected from a Markush group). The term “CNP variant” here is similarly functional as it requires the peptide to be natriuretic.

To illustrate, a claim reciting “a *container* selected from the group consisting of a cup, a bottle, and a box” may have a different scope than “a *drinking vessel* selected from the group consisting of a cup, a bottle, and a box.” The former could refer to any kind of box because all boxes are containers. The latter would require a specialized box, such as milk carton, and not a banker’s box for holding paper files. The genus “drinking vessel” requires the box species to have a structure to support that specified function. Likewise, the genus “enteric polymer” in *Warner Chilcott* was selected from the group consisting of various polymethacrylates, but the term itself was construed to require resistance to drug release in the stomach or ingress of gastric fluids. *See Warner Chilcott*, 2013 WL 1729383, at *8. The Markush genus “pharmaceutically acceptable polymer” in *Abbott* similarly required specific functionality. *Abbott*, 529 F.Supp.2d at 909.

Here, the genus “CNP variant” is not merely a nonce word. It denotes that the listed species have a particular structure to support a particular natriuretic function, as described in the specification. To achieve that function, the structure requires an intact ring between Cys₆ to Cys₂₂ of CNP-22 (and includes any modifications, substitutions, or conjugations). That is, it must support the function of binding to and activating NPR-B, thereby inducing cGMP production.

Put simply, a peptide with the amino acids listed in the Markush group, but without the ring, does not have the structure to bind to and activate NPR-B. Conversely, a molecule that has the relevant amino acid sequence and the ring formed by Cys₆ to Cys₂₂ would not be a “CNP variant” within the meaning of the claims if it has some other component that renders it incapable of binding to NPR-B and activating cGMP. BioMarin’s proposed construction simply ignores the function of the CNP variant in the context of the claimed invention. But one cannot drink from a banker’s box.

B. “hydrolysis” (claims 15–17 and 31–39)

BioMarin opens its brief by suggesting that there is no need to clarify the scope of the term “hydrolysis” because “there can be no reasonable dispute that the accused product can release the active CNP variant by ‘hydrolysis.’” BM Br. at 8. BioMarin knows that statement to be false, as it has already been served with extensive nonburden contentions detailing the legal and factual bases for Respondents’ noninfringement position for this claim limitation. It is important to define hydrolysis for the exact reason that BioMarin wants to avoid doing so: the word “hydrolysis,” as used in the asserted patent, would have been understood by a POSA in 2009 to have one meaning in the context of the specification, and to have a different meaning in other contexts. Those other contexts include the extrinsic evidence describing the accused product that BioMarin cites. *Contra id.* BioMarin’s strategic desire to conflate the uses of that word is the exact situation that claim construction law is designed to guard against. *See Phillips v. AWH Corp.*, 415 F.3d 1303, 1315 (Fed. Cir. 2005) (en banc) (“[T]he specification ‘is always highly relevant to the claim construction analysis. . . . [I]t is the single best guide to the meaning of a disputed term.’” (quoting *Vitronics Corp. v. Conceptronic Inc.*, 90 F.3d 1576, 1582 (Fed. Cir. 1996))).

The term “hydrolysis” appears in claim 15 in the context of “the hydrolysable linkage releas[ing] the CNP variant.” Ex. 1 at 270:64–65. The specification does not define the terms “hydrolysis” or “hydrolysable linkage.” The specification, however, discloses “hydrolysable linkages” only in the context of conjugating a PEG to a CNP variant. EX1001 at 10:37-38; *see also id.* at 60:63-61:19. All uses of the term “hydrolysis” in the specification relate to other reactions—not releasing a CNP variant. Thus, the term “hydrolysis,” as used in the claims, must be read in the specific context of the recited “hydrolysable linkage,” as opposed to other contexts where it may have a broader meaning. *Phillips*, 415 F.3d at 1315. To the extent BioMarin points to other instances of “hydrolysis” in the specification as being probative, those examples are also consistent with Respondents’ construction (as explained in detail below).

Hydrolysis is a term of art in chemistry, and it has a specific chemical meaning consistent with its use by the RE’267 specification. *See Sessler Opening Decl.*, ¶¶ 26-36. That meaning is captured in Respondents’ proposed construction. *Id.* It is true that in some contexts, the term has been used more loosely to describe cleaving chemical bonds without regard to the specific mechanism employed. That is, however, not the plain meaning that “hydrolysis” would have absent a context-specific indication that the term is being used more broadly. For example, in Ascendis’s own later patents (on which BioMarin improperly relies in support of its construction), the applicants acted as their own lexicographers and provided an express, broader definition of “hydrolysis” that departed from the traditional chemical meaning of the term. *Compare* US 11,311,604 B2 at 12:60-67 (“A ‘biodegradable linkage’ or a ‘reversible linkage’ is a linkage that is hydrolytically degradable, i.e. cleavable, in the absence of enzymes under physiological conditions” in “aqueous buffer at pH 7.4.”) *with* BM Br. at 13-14.

The Tsubery reference may also be an example of some authors using hydrolysis more loosely or broadly. *See* BM Br. at 13 (citing Ex. O at 38118). BioMarin appears to cite Tsubery

[REDACTED]

solely because Ascendis cited it in its third-party protest of the reissue application as prior art disclosing a hydrolysable linkage. *Id.* While that document is referenced in the prosecution history, it is no more probative than any other extrinsic evidence as to the meaning of the term “hydrolysis.” *Phillips*, 415 F.3d at 1315-16 (explaining that extrinsic evidence is less probative, if at all). Moreover, neither BioMarin nor the Examiner indicated that they agreed with Ascendis regarding the relevance of Tsubery’s disclosure. A person of ordinary skill reading the prosecution history would not consider Tsubery informative as to the meaning of the term “hydrolysis.” *See* Sessler Reply Decl., ¶¶ 13-17.

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

The relevant question here is how a skilled artisan reading the specification of BioMarin’s patent in 2009 would have understood BioMarin to be using the term in the context of PEG-CNP conjugates—not how others in the art used the term in a different context or how

¹ BioMarin’s reliance (at 14) on the Cleemann reference, U.S. Pat. No. 8,906,847, another Ascendis patent, is even less apposite because Cleemann uses a *different* term, “autohydrolysis.” In Cleemann’s autohydrolysis reaction, water does not react with another substance. *See* Sessler Reply Decl., ¶¶ 18-19. It would not fit Respondents’ or BioMarin’s proposed constructions. *Id.* Cleemann’s reference to “autohydrolysis” is therefore not probative of the plain meaning of “hydrolysis” in the specific context of BioMarin’s patent.

Ascendis sometimes used the term when talking about a different invention or in different contexts years later. *Phillips*, 415 F.3d at 1315-16. BioMarin’s attempt to construe the claims at issue in light of Ascendis’s accused product is legal error. “It is only *after* the claims have been construed *without reference to the accused device* that the claims, as so construed, are applied to the accused device to determine infringement.” *In re Entresto*, 125 F.4th 1090, 1098-99 (Fed. Cir. 2025). Claims must be construed according to how a skilled artisan would have understood the terms at the time the application was filed—not how the patent applicant might have attempted to stretch the terms many years later in view of after-arising technology. *See Kopykake*, 264 F.3d at 1383; *Fundamental Innovation Sys. Int’l LLC v. Anker Innovations Ltd.*, No. CV 21-339-RGA, 2025 WL 459916, at *8 (D. Del. Feb. 11, 2025) (“Terms must be given their meaning at the time of filing.”).

The question before the CALJ is simply whether a skilled artisan reading the specification at the time of the invention would have understood the term “hydrolysis” as being used in its traditional chemical sense or as something broader. To answer that question, one needs to focus chiefly on the intrinsic evidence of BioMarin’s patent, which is consistent with the traditional chemical use of the term. Importantly, the relevant hydrolysis is of a linkage connecting a CNP variant to a synthetic polymeric group, such as a PEG, and the only kinds of linkage mentioned in the specification are amide, imine, aminal, and ester bonds, which can undergo traditional chemical hydrolysis under certain conditions. Ex. 1 at 10:33-43.

BioMarin’s chief intrinsic evidence—three uses of the term “hydrolysis” that it claims would lead a skilled artisan to understand that hydrolysis is being used loosely when referring to the release of CNP variants—do not support BioMarin’s conclusion. BioMarin starts with “deamidation,” which is a kind of natural occurring degradation reaction by which certain amino acids in peptides are slowly converted into other amino acids. *See Sessler Reply Decl.*, ¶¶ 5-6.

The specification discusses measures “to minimize or avoid deamidation of a CNP variant” during manufacture and storage. Ex. 1 at 139:37-40. The specification says that “[b]ecause deamidation occurs by hydrolysis, the substitution of an organic cosolvent for water minimizes contact of the CNP variant with water” and suggests that “[a]lso to minimize or avoid deamidation of a CNP variant, water can be removed from the formulation by lyophilization [i.e., freeze drying].” *Id.* at 139: 40-42, 46-48.

BioMarin admits that deamidation can happen directly by hydrolysis in accordance with Ascendis’s proposed construction (BM Br. at 10 (right-side reaction)), but argues that it can also occur via a multi-step reaction that involves internal ring formation as an intermediate product (*id.*, left-side reaction). BioMarin concedes that Respondents’ construction captures deamidation through the right-hand pathway, which occurs by hydrolysis in *acidic conditions*. BM Br. at 9-11. BioMarin suggests, however, that Respondents’ construction is under-inclusive because it does not capture deamidation by the left-hand pathway that occurs in *physiological conditions*. *Id.* But BioMarin ignores that the context in which the specification seeks to “minimize or avoid deamidation” by hydrolysis is indeed in acidic conditions—not in physiological conditions. Ex. 1 at 139:24-42 (describing the conditions as “a pH of about 3-7, or about 3-6, or about 3.5-6.5, or about 4-6, or about 4-5, or about 4.5-5.5”); *see also* Sessler Reply Decl., ¶ 6 (explaining that the specification’s conditions for deamidation hydrolysis are strictly acidic). Thus, the intrinsic evidence of the meaning of the term “hydrolysis” in the context of deamidation is fully consistent with Respondents’ construction of the term. *See* Sessler Reply Decl., ¶¶ 6-9.

The patent also discusses “acidic hydrolysis” (Ex. 1 at 101:23-27), “NEP hydrolysis” (*id.*, at 113:39-42), and “water-driven hydrolysis” (*id.* at 138:61-63, 139:22-23). BM Br. at 9. These reactions are discussed in different contexts scattered about the patent. None of them refers to releasing a CNP variant. Nonetheless, they are also consistent with Respondents’ definition. *See*

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Sessler Reply Decl., ¶¶ 4, 9. For example, the specification mentions “acidic hydrolysis” in the context of manufacturing peptides in recombinant microorganisms. The resulting peptides are exposed to formic acid to cleave a particular peptide bond in order to achieve peptides of the desired length. Ex. 1 at 101:11-16. Sometimes the acidic environment resulted in additional smaller peptides being created “by excess acidic hydrolysis.” *Id.* at 101:23-27. As explained in Respondents’ opening claim-construction brief, the cleavage of peptide bonds by hydrolysis is consistent with Respondents’ proposed construction—and also with the Examiner’s statement during prosecution that “peptide bonds” are “a type of amide bond, that can be broken by amide hydrolysis, wherein the bonds are cleaved with the addition of a water molecule.” Ex. 23 at 9; Sessler Reply Decl., ¶ 9.

The specification’s mention of “NEP hydrolysis” is similarly consistent with Respondents’ construction because it refers to the cleavage of peptide bonds by the enzyme NEP, using water to break the bonds. Ex. 1 at 113:39-42. Again, the fact that the hydrolysis reaction is catalyzed by an enzyme does not bring it outside Respondents’ construction. *See* Sessler Reply Decl., ¶¶ 10-11. Finally, the specification’s mention of “water-driven hydrolysis” is consistent with Respondents’ construction because the phrase simply denotes a pure hydrolysis reaction that does not involve acid or enzymatic catalysis. Ex. 1 at 138:61-63, 139:22-23. A water molecule cleaves the bond and is added to the product(s) where the bond was broken. *See* Sessler Reply Decl. ¶¶ 10-11. Thus, none of the phrasing in the specification to which BioMarin points would lead a skilled artisan to conclude that the named inventors were using the term “hydrolysis” to mean something broader than its traditional chemical definition, in which the water components are added at the site of cleavage. *Id.*, ¶¶ 4-12.

In sum, BioMarin wants to buck the claim-construction process and jump right to infringement by pointing to extrinsic evidence describing the accused product many years after

the relevant date. BM Br. at 6, 8. That is entirely improper. Claims must be construed “without the objective of capturing or excluding the accused device.” *Vita-Mix Corp. v. Basic Holding, Inc.*, 581 F.3d 1317, 1324 (Fed. Cir. 2009). Indeed, it is inappropriate to even consider evidence describing the accused product when informing claim construction. *See NeoMagic Corp. v. Trident Microsystems, Inc.*, 287 F.3d 1062, 1074 (Fed. Cir. 2002) (“It is well settled that claims may not be construed by reference to the accused device.”) (citing *SRI Int’l v. Matsushita Elec. Corp. of Am.*, 775 F.2d 1107, 1118 (Fed. Cir. 1985) (*en banc*)).² Meanwhile, BioMarin completely ignores the “reference work” that the specification calls out as providing definitions for standard chemistry terms, which supports Respondents’ construction. *See* Res. Br. 18-19. The term “hydrolysis” should be given its plain meaning as a “standard chemistry term” as it would be understood to a skilled artisan reading the specification and Carey in 2009.

C. “coupled to the CNP variant through a hydrolysable linkage” (claims 15–20, 31–48)

All of the arguments discussed above for the “hydrolysis” term apply here as well, because the related term “hydrolysable linkage” should reflect a proper construction for the term “hydrolysis.” *See* Sessler Reply Decl., ¶ 23. That alone is a reason to reject BioMarin’s construction (which incorporates BioMarin’s flawed construction of hydrolysis) in favor of Respondents’ construction.

Additionally, BioMarin’s opening brief does not engage with the fact that a skilled artisan would understand a hydrolysable linkage to be a chemical bond (as opposed to a larger

² BioMarin’s citation of *Wilson Sporting Goods Co. v. Hillerich & Bradsby Co.*, 442 F.3d 1322, 1326-27 (Fed. Cir. 2006) does not counsel otherwise. BM Br. at 6. The “meaningful context” discussed in that case provided an understanding of why the disputed term needed to be construed and whether the proposed construction would resolve the parties’ actual dispute. *Id.* *Wilson* does not stand for the proposition that the accused product itself can be used as evidence of the proper construction.

structure), in the context of the specification and the understanding of a POSA in 2009. *See* Sessler Reply Decl., ¶ 24. All the examples of hydrolysable linkages in the specification are chemical bonds, and what makes those linkages hydrolysable is that the bond is cleaved by an addition of a water molecule in a classic hydrolysis reaction. *Id.*, ¶ 25. That is another reason to reject BioMarin’s construction and adopt Respondents’.

Regarding the capability component of BioMarin’s proposed construction for “coupled... through a hydrolysable linkage,” BioMarin misapprehends Respondents’ proposal. Respondents are not reading into the term “hydrolysable” a requirement that the bond must actually be cleaved at the time of infringement (or ever). But the description of the CNP variant as being coupled to the synthetic polymeric group via a hydrolysable linkage indicates that the decoupling of the CNP variant from the synthetic polymeric group happens by hydrolysis. *See* Sessler Reply Decl., ¶ 26. Respondents’ point is merely that, if the claim specifies “hydrolysable linkage,” then a skilled artisan would understand that language as requiring a bond that undergoes hydrolysis as the mechanism by which it cleaves, as opposed to something that is actually cleaved by some other process that is not hydrolysis. *Id.*

BioMarin’s proposed capability-based construction for “hydrolysable” is a fallback attempt to capture Ascendis’s product, even where a POSA would understand that Ascendis’s CNP is not released by “hydrolysis” as those terms would be understood in 2009. Regardless, whether Ascendis described its linker as “hydrolysable” years after the asserted patent was filed is not probative as a matter of law. *See In re Entresto*, 125 F.4th 1090 (Fed. Cir. 2025); *Kopykake*, 264 F.3d at 1383; *Fundamental Innovation*, 2025 WL 459916, at *8. BioMarin violates basic canons of claim construction by relying on extrinsic evidence describing the accused product rather than confining its analysis to the way a POSA would have understood the claim terms in view of the specification in 2009. *See Vita-Mix*, 581 F.3d at 1324; *NeoMagic*, 287 F.3d at 1074.

That the patent's description of hydrolysable linkages is minimal is not a reason to construe the term more broadly than the specification would support. BioMarin's consequence-driven construction should be rejected.

D. "formulation" (claims 18–20, 40–48)

BioMarin collapses the definitions of "formulation" and "sustained release CNP variant formulation," leaning into its position that the words of the preamble are, not only non-limiting, but meaningless. BioMarin proposes the same construction for both, even though they have dramatically different language and scope. As discussed below for the term "sustained release CNP variant formulation," BioMarin's position is at odds with basic canons of claim construction and conflicts with the prosecution history. The Examiner treated the preamble as a limitation in finding the claims allowable. *See* Res. Br. at 13-14. Specifically, the term "formulation" alone does not connote a sustained-release system. BioMarin's construction should be rejected.

E. "sustained release CNP variant formulation" (claims 18–20, 40–48)

BioMarin first argues that Ascendis "agreed" during prosecution that the preambles were non-limiting. That mischaracterizes the record. To be sure, Ascendis argued in its third-party opposition that the preamble did "not appear" to be limiting. *See* Ex. B at -999 (Ascendis Third Party Protest). What BioMarin neglects to mention is that Ascendis's opposition did not prevail, and the Examiner treated the preamble as a limitation in ultimately allowing the claims to reissue. *See* Ex. 20.

BioMarin then attempts to rebut Ascendis's reliance on the specification's description of sustained-release formulations as physical delivery through excipients by again pointing to Ascendis's *unsuccessful* arguments to the Patent Office during its protest. BM Br. at 21 (citing Ex. B at -999 at 8). That is, again, the opposite of how statements in prosecution history are

treated. Ascendis is not bound to positions on which it *lost*. See, e.g., *SkyHawke Techs., LLC v. Deca Int’l Corp.*, 828 F.3d 1373, 1376 (Fed. Cir. 2016) (“judicial estoppel only binds a party to a position that it advocated and successfully achieved”). Rather, BioMarin is held to the positions on which it *won* to secure the patent. To hold otherwise would give BioMarin the benefit of narrower claims during prosecution and then allow it to broaden them in asserting its patent.

Finally, BioMarin quibbles with the intrinsic support for Ascendis’s construction by observing that it is based on “a single specification passage” describing examples of sustained release formulations. BM Br. at 22. But that “single specification passage” is the *entirety* of the specification’s disclosure of the term “sustained-release formulations.” *Id.* BioMarin does not point to anything else. That the patent’s written description is minimal is not a reason to construe the term more broadly than the specification would support. The sole description of sustained-release formulations identifies them as those known in the art to provide for slow delivery of an API over time using physical (rather than chemical) means to attain the desired release profile. There is no indication in the specification that the molecular structure of the CNP variant construct recited in the body of claim 18 could itself be the source of a sustained-release mechanism.

F. “macromolecule” (claims 15–17, 31–39)

A POSA reading the specification in view of the prosecution history would understand that a “macromolecule” is a functionally active molecule and would understand what that means. Respondents’ construction does not inject any uncertainty. *Contra* BM Br. at 26-27. The functional activity of the macromolecule as described in the specification is the functional activity of the CNP variant. Ex. 25 at 10:9-16. The point of Respondents’ construction is to confirm that the “macromolecule” retains the function of the CNP variant that it contains. It is not an inactive construct that nullifies the activity of the CNP variant within. The specification

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makes clear that preserving the functionality of the CNP variant while in the PEGylated form was the purpose of the invention. Ex. 1 at 9:9-20, 80:19-22. BioMarin's own brief admits that "by arranging the elements as described in the body of claim 15, the half-life of the therapeutic CNP peptide can be extended so as to allow the *active* molecule to exist in the body for a desired time period." BM Br. at 19 (emphasis added). The inventors were looking for ways to extend the half-life of CNP without diminishing its functionality. The PEGylated constructs that the inventors disclose purport to achieve that purpose: extending the half-life of the attached CNP variant without compromising that CNP variant's activity. Ex. 1 at 4:33-35. That is all Respondents' construction requires.

G. "synthetic polymeric group" (claims 15-20, 31-48)

Respondents' construction for "synthetic polymeric group" achieves the same purpose as the construction for macromolecule: staying true to what the inventors identified as their invention. Ex. 1 at 10:33-55. As discussed above, the functional activity of the macromolecule described in the specification is the functional activity of the CNP variant. *See* Section III.A., *supra*. In order for the macromolecule to retain the function of the CNP variant, the synthetic polymeric group must not nullify that function. The specification makes clear that the source of the extended half-life for the CNP variant is the size and placement of the synthetic polymeric group, which is attached outside the ring structure and is small enough to avoid compromising the CNP variant's activity. *See, e.g.*, Ex. 1 at 7:5-39, 9:9-46, 10:33-43, 115:38-43, 61:48-57, Table 4. The synthetic polymeric group should therefore be limited to structures that do not defeat the entire purpose of the invention. That is all Respondents' construction requires.

H. "contacting the/a cell" (claims 21, 22, 49, and 54)

BioMarin's Brief underscores the broad nature of claims 21, 22, 49, and 54. BioMarin does not dispute that "contacting a cell" means "touching the cell itself," consistent with the

plain meaning of the phrase, and as understood and confirmed by one of the named inventors, Joshua Bliesath. But BioMarin improperly adds “including NPR-B” to what a “cell” is, even though claims 22 and 54, for example, do not even require that a “cell” express or include NPR-B (*compare* claims 21 and 22). As BioMarin notes, however, its construction is effectively the same as the simpler construction offered by Respondents, which is “touching the / a cell itself.” BM Br. at 29. BioMarin’s “including NPR-B” adds nothing. Both constructions cover CNP variants stimulating cGMP production (claims 21 and 49) and reversing cell growth arrest (claims 22 and 54) by contacting any part of the cell, including but not limited to NPR-B if cells express it.

BioMarin complains that “Bachem has never identified any support for its contention that the CNP variant recited in the claims must contact something on the cell other than the NPR-B receptor,” BM Br. at 30. This misunderstands Bachem’s position. Bachem does not contend that the CNP *must* contact something on the cell *other than* NPR-B. Bachem’s point is that the claims are broad enough to achieve the claimed result by CNP contacting the cell anywhere.

Bachem’s construction is supported by the portions of the specification BioMarin cites. *See* BM Br. at 30 (citing Ex. 1 at 2:37-54 and 116:5-117:23). Those passages describe CNP binding to and activating NPR-B, but the claims are not limited to binding NPR-B. Indeed, the “cell” of claim 22, for example, need not even express and include NPR-B. The patentee clearly knew how to describe CNP binding to and activating specific receptors, such as NPR-B or NPR-C (Ex. 1 at 116:5-117:23, but chose not to limit the claims in that way.

When applicants know how to use narrowing language but choose not to include it in the claims, that choice has consequences. *See Enzo Biochem, Inc. v. Applera Corp.*, 599 F.3d 1325, 1333 (Fed. Cir. 2010) (“The applicants knew how to claim a linkage group that does not substantially interfere with hybridization...but specifically omitted that language from the

claims.”). BioMarin appears to acknowledge this breadth, as it does not dispute that the claims extend beyond binding to and activating NPR-B. BM Br. at 29-31. But Respondents’ construction better follows the claim language and avoids adding limitations from the specification. Also, testimony supports interpreting “contacting” to mean “touching,” rather than binding to NPR-B. *See* Ex. 31 (10/24/2025 Deposition Transcript of Joshua Bliesath (newly added inventor)) at 220:1–16 (analogizing contacting to touching a table).

Conversely, BioMarin’s construction, “contacting the cell, including NPR-B,” introduces extra wording that is unnecessary and improper. *Deere & Co. v. Bush Hog, LLC*, 703 F.3d 1349, 1354 (Fed. Cir. 2012) (“While claim terms are understood in light of the specification, a claim construction must not import limitations from the specification into the claims.”). The claims themselves illustrate the problem. Claims 22 and 54 apply to any cell that expresses constitutively active FGFR-3. Ex. 1 at 273:15-19 (“22. A method of overcoming cell growth arrest...comprising contacting a cell expressing the constitutively active FGFR-3 with a CNP variant...”). Nothing here suggests that the cells must have an NPR-B receptor. Similarly, BioMarin cites parts of the specification that discuss “downstream signaling of CNP/NPR-B” (BM Br. at 30) and the “proliferation and differentiation of chondrocytes in the growth plate.” (Br. at 30-31). Once again, the claims are not limited to chondrocytes or any type of signaling, and claims 22 and 54 cover cells that lack NPR-B. *Phillips*, 415 F.3d 1303, 1312 (Fed. Cir. 2005) (“[W]e look to the words of the claims themselves . . . to define the scope of the patented invention.”) (quotations omitted). If the applicants had wanted to claim only chondrocytes, or only NPR-B expressing cells, they could have done so. They chose broader language instead. BioMarin’s effort to read NPR-B into the term “contacting the cell” is therefore improper.

IV. CONCLUSION

The CALJ should adopt Respondents’ proposed constructions of the disputed claim terms.

Certificate of Word Count

Pursuant to Ground Rule 7.2, counsel for Respondents certify that this Reply Claim Construction Brief contains 4,954 words. The word count function of Microsoft Word was used to prepare this Reply.

Dated: November 18, 2025

Respectfully submitted,

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**UNITED STATES INTERNATIONAL TRADE COMMISSION
WASHINGTON, D.C.
BEFORE THE HONORABLE CLARK S. CHENEY
CHIEF ADMINISTRATIVE LAW JUDGE**

In the Matter of

CERTAIN DRUG PRODUCTS
CONTAINING C-TYPE NATRIURETIC
PEPTIDE VARIANTS, AND
COMPONENTS THEREOF

Inv. No. 337-TA-1447

DECLARATION OF JONATHAN SESSLER, PH.D.
IN SUPPORT OF RESPONDENTS' REPLY CLAIM CONSTRUCTION BRIEF

I, Jonathan Sessler, hereby declare as follows:

1. The scope of my commission in this matter, my background and qualifications, the materials and legal principles considered, and the bases for my opinions are set forth in my opening declaration previously submitted in the above-captioned Investigation.

2. This Rebuttal Declaration is limited to providing my responses to Dr. Davies's declaration regarding the parties' proposed claim constructions for the terms "hydrolysis" and "coupled . . . through a hydrolysable linkage."

3. In preparing this Rebuttal Declaration, I have reviewed Dr. Davies's declaration and the materials cited therein.

I. Hydrolysis

4. Dr. Davies identifies several examples of hydrolysis reactions disclosed in the specification, including "deamidation occur[ring] by hydrolysis" (Ex. 1 at 139:40), "acidic hydrolysis" (*id.*, at 101:23-27), "NEP hydrolysis" (*id.*, at 113:39-42), and "water-driven hydrolysis" (*id.*, at 138:61-63, Table 16 (footnote), 139:22-23 Table 17 (footnote)). Ex. C ¶ 24. He opines that a POSA would consider these reactions to be hydrolysis. *Id.*, at ¶¶ 24-26. I agree with Dr. Davies that these reactions are examples of hydrolysis, but disagree that any of them are excluded from Respondents' construction. Further, from the passage that Davies cites (Ex. 1 at 138:61-63, Table 16 (footnote)), it is clear the inventors considered hydrolysis and deamidation as separate processes, as they are explicitly separated in their characterization: "hydrolysis, deamidation, isomerization, or cleavage[.]" Ex. 1 at 138:61-63, Table 16 (footnote). A similar distinction is made elsewhere in the patent. *See, e.g.*, Ex. 1 at 139:22-23, Table 17 (footnote).

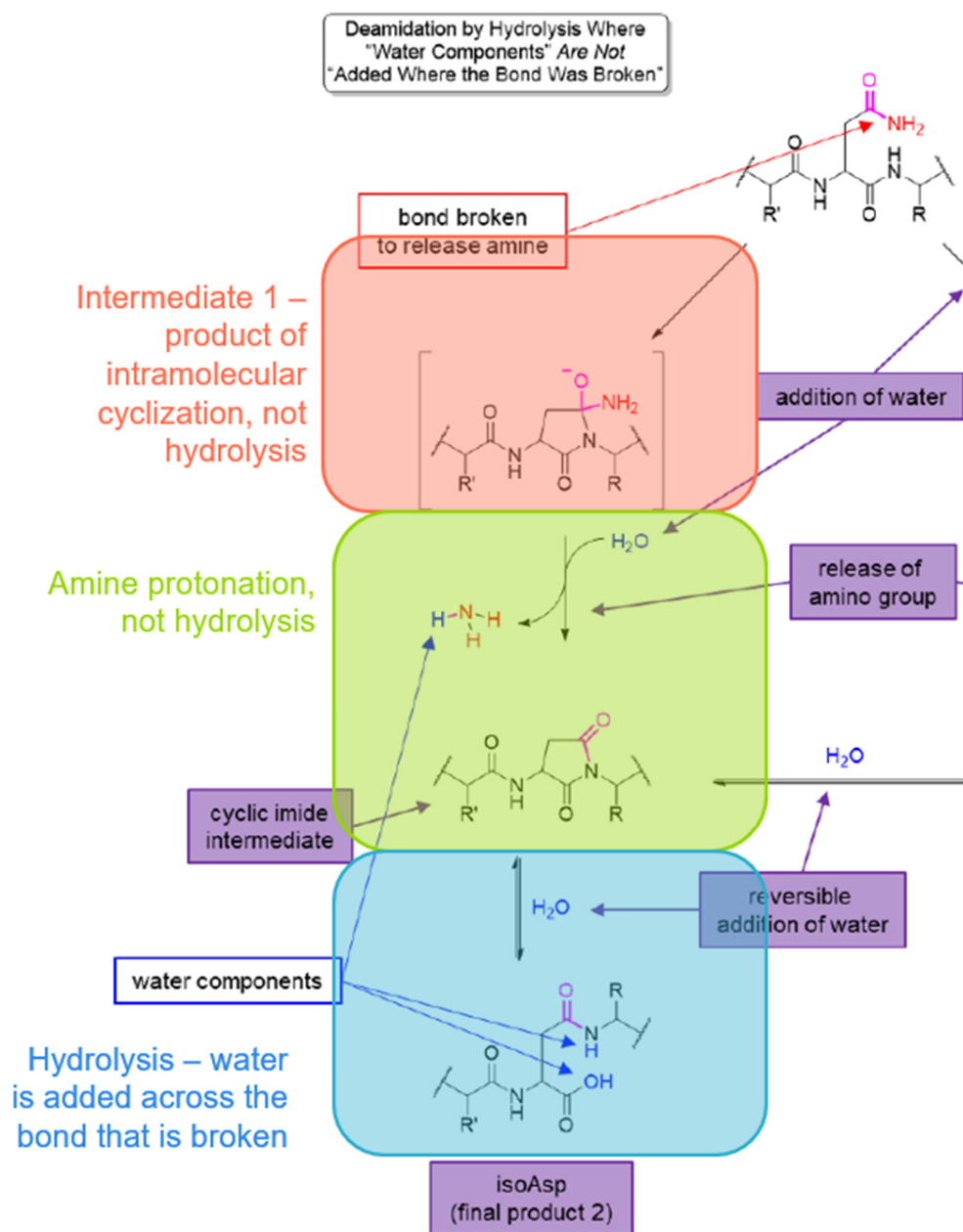
5. The only example that Dr. Davies expands upon is the specification's mention of "deamidation occur[ring] by hydrolysis." Ex. 1 at 139:40-42; Ex. C ¶¶ 25-26. In doing so, he provides a reaction from the literature that he contends is a counterexample to Respondents'

construction. *Id.* (citing Ex. T at 787-793). Dr. Davies illustrates two possible pathways for this reaction, one occurring at physiological conditions (left-hand side) and one occurring in acidic conditions (right-hand side). Ex. C. at FIG. 1. Dr. Davies appears to contend that the right-hand side of the reaction pathway fits Respondents' construction for hydrolysis (and I agree), but the left-hand side does not (I agree that it does not). He concludes that Respondents' construction is therefore incorrect because both reaction pathways are embraced by the specification's reference to "deamidation occur[ring] by hydrolysis." Ex. C at ¶ 25. I disagree with that conclusion.

6. The specification explains that care should be taken to avoid exposing the CNP variants to water, because the water may deaminate the peptide by hydrolysis. Ex. 1 at 139:37-42. Importantly, the preparation and storage conditions of the peptide described are acidic. *Id.*, at 139:24-42 (cautioning one to "minimize or avoid deamidation" during preparation and storage at "a pH of about 3-7, or about 3-6, or about 3.5-6.5, or about 4-6, or about 4-5, or about 4.5-5.5"), *see also id.*, at Table 16, Table 17. A POSA would know that a pH of less than 7 is acidic, and does not reflect the physiological conditions of the claimed peptide constructs in the bloodstream. The deamidation reaction that takes place under these conditions—which the patent cautions one to avoid—would follow the right-hand pathway of Dr. Davies's illustration and not the left-hand side. Ex. C ¶¶ 25-27, FIG. 1.

7. The specification does not state that *all* deamidation reactions necessarily occur by hydrolysis—only that the particular deamidation reaction one should avoid during preparation and storage occurs by hydrolysis. Ex. 1 at 139:40-43. The specification's reference to "deamidation occur[ring] by hydrolysis" therefore only implicates the right-hand pathway which both Dr. Davies and I agree is hydrolysis. *Id.* Furthermore, Patel is clear that, in basic conditions, the deamidation is a separate step from the ensuing hydrolysis. *See* Ex. T at 792 (observing "formation of a cyclic imide intermediate" in neutral and basic pH), Scheme I.

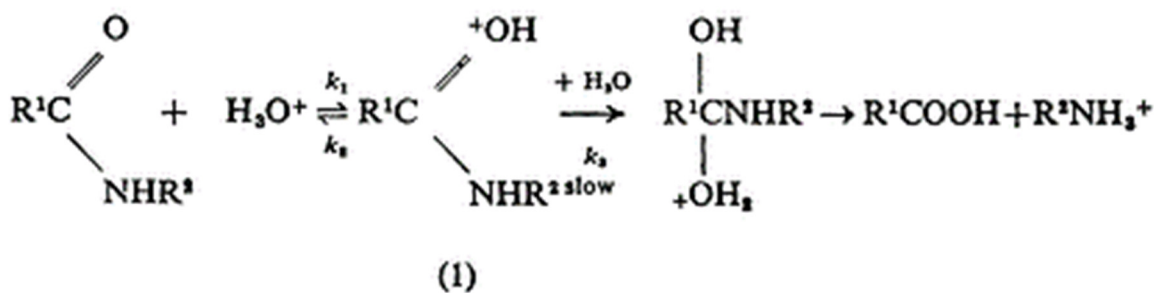
8. I have annotated Dr. Davies's figure below, showing how Dr. Davies erroneously conflates separate steps of a two-step reaction. Here, it is clear that even though hydrolysis may occur later on in a series of reactions, hydrolysis is not inherent to the deamidation reaction. I also note that the "water components" that Dr. Davies points out contain an extra proton—he points to three hydrogen atoms and one oxygen atom in his purported "hydrolysis" example. Ex. C at FIG. 1. This extra H atom comes from a simple protonation of an amine in a previous step in his multiple-reaction scheme, further evidencing that it is a separate acid/base reaction, and not an example of hydrolysis. Respondents' construction of the term hydrolysis is therefore consistent with Patel's figure: deamidation by hydrolysis under acidic aqueous conditions contains a protonation step, followed by a hydrolysis step consistent with Respondents' construction. Ex. T at Scheme I.



Ex. C at Figure 1 (annotated).

9. Dr. Davies does not specifically explain how the other examples of hydrolysis in the specification might be inconsistent with Respondents' construction, but for completeness I will walk through them. First, the specification's mention of "acidic hydrolysis" is also consistent with Respondent's construction, as it is the reaction between a compound and water, catalyzed by an acid, where a bond is cleaved and a constituent H^+ and OH^- are added where the

bond was broken. Ex. 1 at 101:23-27. This is illustrated by the “acid hydrolysis” mechanism disclosed by O’Connor in 1970, shown below. That an acid (or acidic environment) is also involved does not exclude the reaction from Respondents’ construction.



Scheme 1

Ex. 28 at Scheme 1.

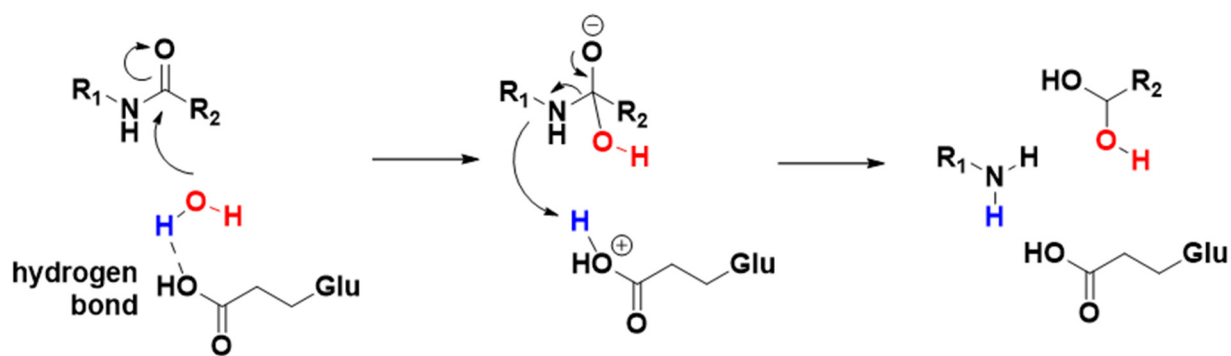
10. The specification’s mention of “NEP hydrolysis” is similarly consistent with Respondents’ construction because it refers to the cleavage of peptide bonds by the enzyme NEP, using water to break the bonds. Ex. 1 at 113:39-42. As disclosed by Devault *et al.*, NEP “hydrolyses peptide bonds” and describes the mechanism by comparing it to a similar protein, thermolysin:

According to Hangauer *et al.* (1984), Glu-143 of thermolysin plays a major role in catalysis. First, as a base catalyst, it triggers the nucleophilic attack of a water molecule on the carbonyl group of the scissile peptide bond. It also acts as a proton shuttle between the water molecule and the nitrogen of the amide bond to be cleaved. The observation that recombinant ***neutral endopeptidase enzymes containing mutations at Glu-584 are virtually devoid of activity strongly suggests a similar role for this residue.***

Ex. 29 at 4040 (emphasis added).

11. A POSA would understand that “nucleophilic attack of a water molecule on the carbonyl group” results in the addition of OH to one side of the bond to be broken (the “scissile” bond), and H to the other side via a “proton shuttle.” *Id.* One probable mechanism for this

transformation is depicted below.¹ Further, structural studies of the NEP protein reveal that an amino acid in the active site “is in position to be involved in catalysis by polarizing the attacking water molecule.” Ex. 30 at 345-46; *see also* Ex. 29 at 4040. A POSA would understand that the “attacking water molecule” is used for hydrolysis in a manner consistent with Respondents’ construction. Ex. 30 at 346; *see also* Ex. 29 at 4040. The fact that the hydrolysis reaction is catalyzed by an enzyme does not bring it outside Respondents’ construction and would not change a POSA’s appreciation that the cleavage involves hydrolysis.



12. I also note that the specification’s mention of “water-driven hydrolysis” is consistent with Respondents’ construction because the phrase simply denotes a pure hydrolysis reaction that does not involve acid or enzymatic catalysis. Ex. 1 at 138:61-63, 139:22-23. A water molecule cleaves the bond and is added to the product(s) where the bond was broken. Accordingly, Respondents’ construction does not exclude any examples of hydrolysis disclosed in the specification. It is entirely consistent with the specification and how a POSA in 2009 would interpret the term hydrolysis.

¹ A POSA would also understand that the subsequent amine and carboxylic acid products of this transformation might undergo additional proton transfer reactions, as per typical acid-base chemistry.

13. Dr. Davies next discusses the hydrolysis reaction in Tsubury, as a purported counterexample to Respondents' proposed construction. Ex. C ¶¶ 28-31. I disagree with Dr. Davies that Tsubury discloses an example of a hydrolysis reaction. Ex. O at 38118. As a preliminary matter, it appears that Dr. Davies points to Tsubury only because Ascendis cites it as an example of a hydrolysable linker during prosecution. *Id.* There is no indication that BioMarin or the Examiner agreed with Ascendis as to what Tsubury would teach a skilled artisan about hydrolysis or hydrolysable linkers. I note that Tsubury states that the linker disclosed can undergo "spontaneous chemical hydrolysis," but it does not explain what is meant by that phrase. (Ex. O at 38118).

14. There is one reaction illustrated in Tsubury, but that reaction does not appear to show hydrolysis. *Id.*, at 38119. Indeed, there is no indication that water is involved at all in that reaction. Dr. Davies takes this reaction and annotates it with water molecules to hypothesize how the reaction might proceed by hydrolysis. Ex. C at FIG. 3. I note that Tsubury itself does not disclose these water molecules or provide any context from which a POSA might envision their involvement. Dr. Davies is an expert in the field and can draw upon his exceptional experience and creativity to hypothesize obscure reactions that would not be understood by a person of ordinary skill reading Tsubury.

15. To that end, Dr. Davies's annotated figure reflects his own hypothesis about how water molecules might move through the reaction shown in Tsubury. But a POSA would know that the water molecules he has added are neither necessary nor sufficient to cause the elimination reaction discussed in Tsubury to occur. Indeed, while it is possible that water acts as a base to cause the reaction to occur, it is my opinion that a POSA, upon reading the specification, claims, and prosecution history of the RE'267 patent, would not envisage base-promoted elimination to be encompassed by the scope of "hydrolysis." Rather, a POSA would

understand that water must be a necessary reactant for a reaction to correctly be described as a hydrolysis reaction.

16. Further, it is clear that Dr. Davies invokes a chemical mechanism that is implausible to the point of being nonsensical in an attempt to incorporate the components of water into the reaction products of Tsubury. Tsubury's release mechanism is a mass-balanced reaction—no atoms are added or removed from the reaction. Rather, a base promotes a proton transfer from the linker to the released peptide. No atoms are gained or lost in this reaction. Dr. Davies stretches the limits of chemical possibility by showing a weak base – hydroxide – subsequently attacking a methylene to produce a carbanion. Conversion from a weak base (OH^-) to a strong base (R_3C^-) is close to chemically impossible in this context. I suspect that Dr. Davies proposes this tortured mechanism so that he can inappropriately fit it into Complainant's definition of "hydrolysis."

17. In my opinion, a person of ordinary skill reading the prosecution history might consult Tsubury because Ascendis suggested that it discloses an example of a hydrolysable linkage that is relevant to the claims. But upon reading Tsubury, a person of ordinary skill would not understand Tsubury to illustrate any hydrolysis reactions. A person of ordinary skill would not redefine their understanding of the plain meaning of hydrolysis to include the reaction disclosed in Tsubury simply because a third-party cited the article as potentially relevant. Rather, a person of ordinary skill would read Tsubury's reference to "spontaneous chemical hydrolysis" (Ex. O at 38118) as being a confusing turn of phrase that is being used loosely, and which sheds little light on what is actually happening when the linker molecule breaks down.

18. Dr. Davies next identifies one of Ascendis's patents from around 2009, "Cleemann," as an example of usage in the art of the term hydrolysis. Ex. C ¶¶ 32-36. I disagree with him that this is a relevant counterexample to Respondents' construction. Cleemann does not

use the term “hydrolysis.” Cleemann uses a *different* term, “autohydrolysis.” Ex. P at 4:7-11.

Despite having the root word “hydrolysis” in its name, the “autohydrolysis” release mechanism depicted by Cleemann does not implicate water as a reactant. It is not hydrolysis.

19. The so-called “autohydrolysis” described in Cleemann would not satisfy *either* party’s proposed construction for “hydrolysis.” This is because water does not “react with another substance” in the release step described in Cleemann. Therefore, I do not think a POSA in 2009 would consider Cleemann’s reference to “autohydrolysis” to reflect the plain meaning of the unmodified term “hydrolysis” in the context of BioMarin’s patent, which uses that word consistently with its standard chemistry definition.

20. I note that BioMarin has also cited a few documents that purport to describe Ascendis’s accused product using the term “hydrolysis.” *See* BM Opening Exhibits I, J, L. At least two of them do not appear to be publicly available. I have reviewed these documents, but they do not change my opinion on how a POSA in 2009 would understand the term in the context of the RE’267 patent. These documents post-date the RE’267 patent by many years and do not appear to be using the term “hydrolysis” in a way that is precise or consistent with its ordinary meaning in chemistry in 2009 or even today.

21. Finally, I note that Dr. Davies does not address the example of ring-opening hydrolysis in his declaration, which is irreconcilable with BioMarin’s proposed construction. Specifically, a bond may be cleaved by hydrolysis *without* forming “two or more new substances.” Sessler Opening Decl. ¶ 34-36. I provided a few examples of ring-opening hydrolysis in my opening declaration, including a succinimide ring and an epoxide. *Id.*

22. Accordingly, even in view of Dr. Davies’s declaration, it remains my opinion that Respondents’ construction for “hydrolysis” best captures the plain meaning that the term “hydrolysis” would have to a POSA in the context of the RE’267 specification in 2009.

II. Coupled to the CNP variant through a hydrolysable linkage

23. My opinions discussed above for the “hydrolysis” term apply here as well, because the related term “hydrolysable linkage” should reflect a proper construction for the term “hydrolysis.” Respondents’ proposed construction for “coupled to the CNP variant through a hydrolysable linkage” does that, while BioMarin’s competing proposal does not.

24. I note that Dr. Davies’s declaration does not address whether a POSA would understand a hydrolysable linkage to be a chemical bond (as opposed to a larger structure), in the context of the specification and the understanding of a POSA in 2009. All of the examples of hydrolysable linkages in the specification are chemical bonds, and what makes those linkages hydrolysable is that the bond is cleaved by addition of a water molecule in a classic hydrolysis reaction. This is another reason I believe that Respondents’ proposed construction better captures the meaning of “hydrolysable linkage” as used in the claim.

25. The specification discloses “hydrolysable or stable linkage[s],” as “amide, imine, aminal, alkylene, or ester *bond*,” and mentions Sunbright CS as a commercial example. Ex. 1 at 10:37-39 (emphasis added); Ex. 12. To the extent these linkages are hydrolysable,² the hydrolysis proceeds according to Respondents’ construction: the linkage is cleaved, and constituents of a water molecule are added where the bond was broken. Thus, all “hydrolysable linkages” described in the RE’267 patent are consistent with Respondents’ construction, which reflects the plain meaning the term would have to a POSA in 2009.

26. I note that Dr. Davies interprets the term “hydrolysable” to require only a capability of being hydrolyzed. Ex. C ¶ 39. I do not disagree but maintain my opinion that by specifying “hydrolysable linkage” the claim communicates to a POSA that the bond coupling the

² Not all linkages are hydrolysable (*e.g.*, alkylene bonds are not hydrolysable). Some linkages are hydrolysable only under certain conditions.

CNP variant to the synthetic polymeric group undergoes hydrolysis as the chemical process by which it is cleaved. A POSA would not consider a linkage that is cleaved by some *other* process in the presence of water, and hydrolyses only under *extreme* conditions, to be a hydrolysable linkage. Indeed, the Carey reference incorporated by reference to the RE'267 patent, and thus intrinsic evidence to claim construction, states that "[t]he hydrolysis of amides to carboxylic acids and amines requires considerably more vigorous conditions than ester hydrolysis." Ex. 4 at 473. As such, a POSA would understand that even though functional groups such as amide bonds are capable of hydrolysis under the "vigorous" conditions such as extremely basic environments, does not mean that a POSA would consider such bonds to be hydrolysable in the context of the RE'267 patent.

27. For these reasons, and the reasons in my opening declaration, Respondents' construction is most faithful to the way a POSA would understand molecular structures to be coupled through a hydrolysable linkage in the context of the RE'267 specification in 2009.

III. CONCLUSION

28. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true.

Executed on this 14th day of November, 2025, in Austin, TX.



JONATHAN L. SESSLER, PH.D.

EXHIBIT 28

Acidic and Basic Amide Hydrolysis

By Charmian O'Connor

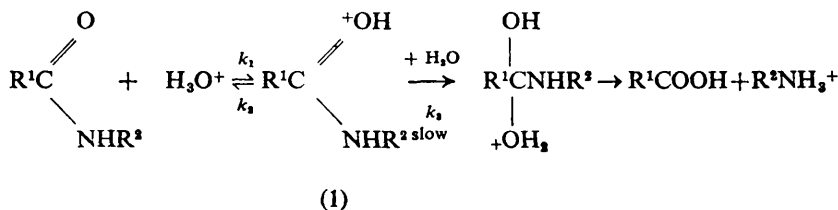
CHEMISTRY DEPARTMENT, UNIVERSITY OF AUCKLAND, NEW ZEALAND

The rates of acid catalysed reactions depend on acid concentration. Most reactions are accelerated, and some rates reach a maximum, while others pass through a maximum and then decrease.^{1,2} Recently it has been shown that this behaviour may be further complicated as some reactions which have a rate maximum at intermediate acid concentrations may decrease to a minimum and then increase in rate at high acid concentrations.^{3,4} Amides exhibit all these patterns of behaviour and attempts have been made to explain them. These explanations have usually been based on small amounts of data.

The mechanism of basic amide hydrolysis is straightforward, and recent investigations have interpreted substituent, catalytic, and isotope effects.

1 Acid Hydrolysis

A. Substituent Effects.—The mechanism generally accepted (Scheme 1) for the hydrolysis of amides in dilute acid solution⁵ involves attack by a water molecule on the conjugate acid of the amide (1) as the rate determining step.



Scheme 1

Reid⁶ studied the acid catalysed hydrolysis of amides and showed that the rate was first order in both total amide and hydrogen ion concentrations, at least in reasonably dilute solutions. He also investigated the effect of substituents on the hydrolysis rates of substituted benzamides, and found there were no marked

¹ J. F. Bunnett, *J. Amer. Chem. Soc.*, 1961, **83**, 4956; 4968; 4973; 4978.

² C. K. Ingold, 'Structure and Mechanism in Organic Chemistry', Cornell University Press, Ithaca, N.Y., 1953.

³ D. Jaques, *J. Chem. Soc.*, 1965, 3874.

⁴ K. Yates and R. A. McClelland, *J. Amer. Chem. Soc.*, 1967, **89**, 2686.

⁵ M. L. Bender, *Chem. Rev.*, 1960, **60**, 53.

⁶ E. E. Reid, *Amer. Chem. J.*, 1899, **21**, 284; *ibid.*, 1900, **24**, 397.

Acidic and Basic Amide Hydrolysis

effects for *meta*- and *para*- substituents, but *ortho*- substituents retarded the rate. Leisten⁷ found that when substituted benzamides were hydrolysed in concentrated perchloric acid solutions electronic effects occurred, and that electron-attracting groups accelerated, while electron-donating groups retarded, the rate. (Relative rates for hydrolysis of *p*-nitrobenzamide, benzamide, and *p*-toluamide in 7.19M HClO₄ at 95 °C were 1.40, 0.228, and 0.129 hr⁻¹.) Meloche and Laidler⁸ found that the activation energy for hydrolysis of substituted benzamides in an ethanol-water mixture was raised by electron-attracting, and lowered by electron-donating, substituents. Bruylants and Kezdy⁹ summarised results and suggested that increasing chain length in aliphatic R groups retarded the rate (*n*-butyramide twice as slow as propionamide), but halogen substituents enhanced the rate (trichloroacetamide twice as fast as chloroacetamide). Their conclusion was that polar effects are not as important as steric hindrance either by increased chain length or by *ortho*- substituents in substituted benzamides. Koshy¹⁰ demonstrated the marked stabilising effect of the hydroxy-group in the *ortho*- position when he compared the stability of benzamide, salicylamide, and some *N*-substituted derivatives. Bolton and co-workers,¹¹ studying the hydrolysis of primary aliphatic amides in dilute acids, found that the hydrolysis was governed by a combination of steric and conjugative substituent effects.^{11a} Their data for halogen-amides^{11b} showed that amides might be slightly sensitive to polar effects.

B. Occurrence of a Rate Maximum.—Since the acid hydrolysis of amides is reasonably slow, reactions may be carried out in quite high acid concentrations, and as the acid concentration is increased the hydrolysis rate increases to a maximum and then decreases. Benrath¹² was the first to discover this phenomenon and subsequent workers^{13–16} indicate that it generally occurs. The position of the maximum appears to differ with respect to hydrogen ion concentration both with different amides and different mineral acids.¹⁶

C. Deuterium Isotope Effect.—Reitz¹⁵ and Bunton, O'Connor, and Turney¹⁷ have studied the deuterium isotope effect on the acid hydrolysis of acetamide and benzamide respectively in protium and deuterium oxide. Both investigations showed that the overall effect was to shift the entire rate of acidity profile so that the maximum occurred at lower hydrochloric acid concentration in D₂O than in

⁷ J. A. Leisten, *J. Chem. Soc.*, 1959, 765.

⁸ I. Meloche and K. J. Laidler, *J. Amer. Chem. Soc.*, 1951, 73, 1712.

⁹ A. Bruylants and F. Kezdy, *Rec. Chem. Progr.*, 1960, 21, 213.

¹⁰ K. V. Koshy, *J. Pharm. Sci.*, 1969, 58, 560.

¹¹ (a) P. D. Bolton and I. R. Wilson, *Austral. J. Chem.*, 1965, 18, 795; (b) P. D. Bolton, *Austral. J. Chem.*, 1966, 19, 1013; (c) P. D. Bolton and G. L. Jackson, *Austral. J. Chem.*, 1969, 22, 527.

¹² A. Benrath, *Z. anorg. Chem.*, 1926, 151, 53.

¹³ H. V. Euler and A. Olander, *Z. phys. Chem. (Leipzig)*, 1927, 131, 107.

¹⁴ T. W. J. Taylor, *J. Chem. Soc.*, 1930, 2741.

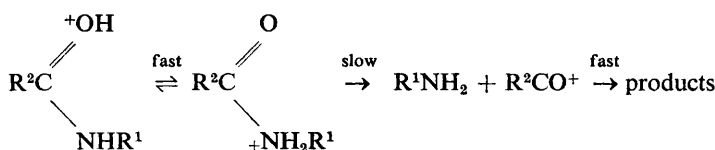
¹⁵ O. Reitz, *Z. phys. Chem. (Leipzig)*, 1939, 183, 371.

¹⁶ V. K. Krieble and K. A. Holst, *J. Amer. Chem. Soc.*, 1938, 60, 2976.

¹⁷ C. A. Bunton, C. J. O'Connor, and T. A. Turney, *Chem. and Ind.*, 1967, 1835.

H₂O. Bell¹⁸ and Wiberg¹⁹ account for this by stating that D₂O is a weaker base than H₂O because its ionic product is one fifth that of water. Hence for a reaction involving a substrate only partially protonated in a rapid pre-equilibrium, the rate determining step will occur faster in D₂O than in H₂O, *i.e.* $k_D/k_H > 1$. In higher acidities the substrate is essentially entirely protonated, and reaction of the protonated substrate becomes important in the kinetic rather than the equilibrium step. Since D₂O is less nucleophilic than H₂O a ratio $k_D/k_H < 1$ is observed. Both these observations and Arrhenius parameter work^{20–22} are in accord with a bimolecular mechanism.

D. Unimolecular Mechanism.—During hydrolysis of the nitro-derivatives of acetanilide in sulphuric acid^{22a,b,23} a more complex dependence of k_ψ (the pseudo first-order rate constant) on acid concentration was observed, *viz.*, a rate maximum at intermediate acidities followed by a minimum and subsequently increasing rate at high acid concentration. Duffy and Leisten²³ discuss their results in terms of a change in mechanism from $A_{Ac}2$ to $A_{Ac}1$. Similar rate profiles have more recently been observed for esters^{3,4} and ethyl carbamate.²⁴ It is proposed²³ that Scheme 2 occurs above 70% w/w sulphuric acid,



Scheme 2

and in a unimolecular mechanism the position of protonation is important because dissociation of the oxygen-protonated form cannot seriously be considered to explain the reaction in highly acidic media.

Vinnik^{22b} agrees that the mechanism changes from bimolecular to unimolecular at *ca.* 70% w/w sulphuric acid but differs in his explanation of the increasing rate of hydrolysis. He assumes that in concentrated solutions of sulphuric acid the protonated amide and molecules of undissociated sulphuric acid react. The activated complex incorporates the AH⁺ ion and the sulphuric acid molecule, whereas in the bimolecular mechanism at low acidities it incorporates the amide, water molecules, and a proton.

¹⁸ R. P. Bell, 'Acid Base Catalysis', Oxford University Press, 1941.

¹⁹ K. B. Wiberg, *Chem. Rev.*, 1955, **55**, 713.

²⁰ B. S. Rabinovitch and C. A. Winkler, *Canad. J. Res.*, 1942, **20**, B, 73.

²¹ H. H. G. Jellinek and A. Garden, *J. Phys. and Colloid Chem.*, 1949, **53**, 996; H. H. G. Jellinek and J. R. Urwin, *J. Phys. Chem.*, 1953, **57**, 900.

²² (a) M. I. Vinnik, I. M. Medvetskaya, L. R. Andreeva, and A. E. Tiger, *Russ. J. Phys. Chem.*, 1967, **41**, 128; (b) M. I. Vinnik and I. M. Medvetskaya, *Russ. J. Phys. Chem.*, 1967, **41**, 947; (c) M. I. Vinnik and I. M. Medvetskaya, *Russ. J. Phys. Chem.*, 1969, **43**, 345.

²³ J. A. Duffy and T. A. Leisten, *J. Chem. Soc.*, 1960, 853.

²⁴ V. C. Armstrong and R. B. Moodie, *J. Chem. Soc. (B)*, 1969, 934.

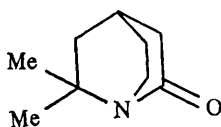
Acidic and Basic Amide Hydrolysis

E. Position of Protonation.—Several authors^{8,9,25,26} have suggested that the acid-catalysed hydrolysis of amides may go *via* either of two mechanisms involving distinct intermediates and transition states, depending on whether the oxygen or nitrogen of the amide has been protonated. The following indicates some of the controversies regarding the position of protonation.

(i) *Theoretical considerations.* The usual justification for considering oxygen-protonation is that amides are mesomeric.²⁶ Proton addition could occur at the atom carrying the partial negative charge. Moreover, *O*-protonated amide ions are themselves mesomeric, unlike the *N*-protonated cations. Protagonists of nitrogen-protonation²⁷ have argued that the mesomeric stabilities would not be important enough to outweigh the inherently greater basicity of nitrogen over oxygen.

Theoretical calculations have been made²⁸ on the protonation in the gas phase of various carboxylic acid derivatives. The opposite site of protonation from that observed experimentally in solution was predicted for compounds containing two heteroatoms. Allowances made for stabilisation by solvation of the oxygen- but not the nitrogen-protonated forms of formamide were insufficient to reverse the order of energies of the two conjugate acids. The authors used as partial support for their prediction of nitrogen-protonation the lack of oxygen exchange^{29,30} between benzamide and H_2^{18}O in acid solution. However, Bender²⁹ explains that this result is not inconsistent with oxygen-protonation if in Scheme 1 $k_3 \gg k_2$.

(ii) *Chemical evidence.* Trialkylammonium salts are readily hydrolysed,³¹ whereas amide hydrolysis is relatively difficult. Klages and Zange³¹ considered this as evidence that the major component of the protonated amide was the *O*-protonated form, but that acid hydrolysis occurred through the *N*-protonated species. This interpretation was supported by Pracejus,³² who made the amide (2) and



(2)

found that it is rapidly hydrolysed. In (2) the lone pair of electrons on the nitrogen atom is in an orbital perpendicular to the π orbital of the $>\text{C}=\text{O}$ group,

²⁵ J. T. Edward and S. C. R. Meacock, *J. Chem. Soc.*, 1957, 2000; 2007; 2009.

²⁶ R. Huisgen and H. Brade, *Chem. Ber.*, 1957, **90**, 1432.

²⁷ J. T. Edward, H. S. Chang, K. Yates, and R. Stewart, *Canad. J. Chem.*, 1960, **38**, 1518.

²⁸ A. C. Hopkinson, R. A. McClelland, K. Yates, and I. G. Csizmadia, *Theor. Chim. Acta*, 1969, **13**, 65.

²⁹ (a) N. L. Bender and R. D. Ginger, *J. Amer. Chem. Soc.*, 1955, **77**, 348; (b) M. L. Bender, Y. L. Chow, and F. Chloupek, *J. Amer. Chem. Soc.*, 1958, **80**, 5380.

³⁰ C. A. Bunton, T. A. Lewis, and D. R. Llewellyn, *Chem. and Ind.*, 1954, 1154.

³¹ V. F. Klages and E. Zange, *Annalen*, 1957, **607**, 35.

³² H. Pracejus, *Chem. Ber.*, 1959, **92**, 988.

thus preventing mesomerism, and it is likely that a *N*-protonated cation is formed.

(iii) *Basicities*. Huisgen and Brade²⁶ presented evidence pointing to the predominance of *O*-protonation. They found that *N*-substituents have less effect on the basicities of amides than on those of corresponding amines, suggesting that the basic centre of amides is further from the nitrogen atom than in amines.

However, application²⁷ of the Hammett equation³³ to basicities of *meta*- and *para*-substituted benzamides led to better correlation of pK_{AH}^+ with σ than with the σ^+ values required for protonation of the carbonyl oxygen of carboxyl groups,³⁴ suggesting, therefore, that amides protonate on nitrogen. However, the authors note that care is necessary in making this comparison, since the concept that all reactions comply with a set of unique σ or σ^+ values has been criticised.³⁵

(iv) *Ultraviolet spectra*. Hantzsch³⁶ used the similarities in u.v. spectra of benzamide and ethylbenzamido-ether to suggest *O*-protonation. Edward *et al.*,²⁷ however, showed that the evidence was also compatible with *N*-protonation. The spectrum has also been interpreted in terms of two equilibria,³⁷ of which the first was assumed to involve *O*-protonation, and the second (occurring at low acidities) involved resonance of electronic structures. The hypsochromic shift observed for thioacetamide³⁸ was favoured as evidence of *N*-protonation.

(v) *Infrared and Raman spectra*. Evidence from these sources, *e.g.* shifts in frequency,³¹ has generally been interpreted in terms of *N*-protonation, but Katritzky and Jones³⁹ state that some assumptions were not justified, and they do not consider that the evidence negates the possibility of *O*-protonation.

(vi) *Proton magnetic resonance*. The strongest support for *O*-protonation comes from ¹H n.m.r. Gillespie and Birchall⁴⁰ slowed down the rate of exchange of protons of acetamide, *NN*-dimethylacetamide, formamide, and dimethylformamide with the solvent by lowering the temperature and unambiguously assigned the spectra to the C=OH⁺ group. The spectrum of pure liquid dimethylformamide⁴¹ shows two peaks for the two methyl groups, which have different environments about the OC—NMe₂ bond as a result of its partial double bond character. These peaks remain unchanged in aqueous strong acids.⁴² It is assumed then that the *O*-protonated form predominates in strong acid solution,

³³ L. P. Hammett, 'Physical Organic Chemistry', McGraw-Hill, New York; 1940, p. 184.

³⁴ R. Stewart and K. Yates, *J. Amer. Chem. Soc.*, 1960, **82**, 4059.

³⁵ H. van Bekkum, P. E. Verkade, and B. M. Wepster, *Rec. Trav. chim.*, 1959, **78**, 815.

³⁶ A. Hantzsch, *Ber.*, 1931, **64**, 667.

³⁷ A. R. Goldfarb, A. Mele, and N. Gutstein, *J. Amer. Chem. Soc.*, 1955, **77**, 6194.

³⁸ H. Hosoya, J. Tanaka, and S. Nagakura, *Bull. Chem. Soc. Japan*, 1960, **33**, 850.

³⁹ A. R. Katritzky and R. A. Jones, *Chem. and Ind.*, 1961, 722.

⁴⁰ R. J. Gillespie and T. Birchall, *Canad. J. Chem.*, 1963, **41**, 148.

⁴¹ W. D. Phillips, *J. Chem. Phys.*, 1955, **23**, 1363; H. S. Gutowsky and C. H. Holm, *ibid*, 1956, **25**, 1228.

⁴² G. Fraenkel and C. Franconi, *J. Amer. Chem. Soc.*, 1960, **82**, 4478.

Acidic and Basic Amide Hydrolysis

because the NHMe_2 group should be free to rotate. Similar results have been obtained with other amides.^{43*}

(vii) *Conclusion.* It appears that the *O*-protonated form predominates but that *N*-protonation occurs to a small degree. Thus it is reasonable to postulate hydrolysis mechanisms involving a rapid protonation pre-equilibrium on either the oxygen or nitrogen atom. Acid dissociation constants in the literature are assumed to refer to the *O*-protonated conjugate acid. The *N*-protonated forms will be strong acids and will therefore exist only in small concentrations even in concentrated acid solutions.

F. Amide Acidity Function.—The original H_0 function has recently been recognised as having less applicability than was formerly supposed.⁴⁴ Yates⁴⁵ extended the idea of having a specific acidity function for a particular structural type of base to amides, by producing the H_A function, which is analogous to H_0 , but applicable to the ionisation of amides by proton addition. H_A is based on a set of primary substituted benzamides, except in dilute acids where there is no substituted benzamide basic enough to be considerably protonated, so it is initially based on *p*-nitroaniline as the primary indicator. Bunnett and Olsen⁴⁶ noted this uncertainty and suggest that the whole function is too negative by *ca.* 0.3 units, and this will affect $\text{p}K_{\text{AH}^+}$ values determined using H_A . The values have been measured in sulphuric^{45a} and hydrochloric^{45c} acids and the function tested^{45b} for several amides other than those used originally, with generally good agreement.

Yates and Stevens^{45b} stress that rate-acidity correlations of the Zucker-Hammett⁴⁷ or Bunnett *w*-function¹ types lose much significance if the correct acidity function for the particular base type being studied is not used.

G. Explanation of Rate Maximum.—Various attempts have been made to interpret the maximum and subsequent decrease in the acid hydrolysis rate profile. Benrath⁴⁸ suggested that the rate maximum occurred at the point of complete ionisation and the subsequent decrease might be due to complex salt formation. Taylor¹⁴ thought this suggestion unlikely since the position of the maximum was independent of the amide concentration, and suggested that the mechanism might

* Recently, however, this technique has provided the first direct proof of an equilibrium concentration of an *N*-protonated amide.

S. R. de Lockerente, O. B. Nagy, and A. Bruylants, *Org. Mag. Resonance*, 1970, **2**, 179, studied the protonation of five substituted anilides and in the case of 2,4 dinitroformanilide a new peak discovered at δ equals 8.25 was attributed to the formyl proton undergoing a diamagnetic displacement by reason of *N*-protonation, occurring because of enhanced basicity of the nitrogen atom.

⁴³ A. Berger, A. Loewenstein, and S. Meiboom, *J. Amer. Chem. Soc.*, 1959, **81**, 62.

⁴⁴ E. M. Arnett, *Progr. Phys. Org. Chem.*, 1963, **1**, 233.

⁴⁵ (a) K. Yates, J. B. Stevens, and A. R. Katritzky, *Canad. J. Chem.*, 1964, **42**, 1957; (b) K. Yates and J. B. Stevens, *Canad. J. Chem.*, 1965, **43**, 529; (c) K. Yates and J. C. Riordan, *Canad. J. Chem.*, 1965, **43**, 2328.

⁴⁶ J. F. Bunnett and F. P. Olsen, *Canad. J. Chem.*, 1966, **44**, 899.

⁴⁷ L. Zucker and L. P. Hammett, *J. Amer. Chem. Soc.*, 1939, **61**, 2791.

⁴⁸ A. Benrath, *Z. anorg. Chem.*, 1926, **151**, 53.

change as the acid concentration increased. It was further suggested¹⁶ that the decrease in rate was due to decreasing water activity. Ingold² explained that an increase in solute concentration reduces water activity, and when this effect was proportionally greater than its effect in enhancing acidity, the rate of hydrolysis fell. Edward and Meacock²⁵ accepted this explanation and extended it slightly to obtain an equation (1) which gave satisfactory theoretical rate-acidity profiles up to the maximum.

$$k_{\psi} = k K_{\text{AH}^+} [\text{H}_3\text{O}^+] / (K_{\text{AH}^+} + h_0) \quad (1)$$

They regarded the hydrolysis of amides as a bimolecular process with the equation for the rate of the limiting stage including the ionised form of the reactant and water. They also suggested that the intermediate could be either the *O*- or *N*-protonated form, both forms leading to kinetically indistinguishable results. Vinnik *et al.*^{22a} used equation (2) for that portion of the profile up to a minimum in the hydrolysis of *o*-nitroacetanilide,

$$k_{\text{eff}} = k C_{\text{H}_3\text{O}^+} / (1 + h_0/K_B) \quad (2)$$

where k_{eff} is the effective rate constant, K_B is the acid dissociation constant, and k is the rate constant corrected for the degree of protonation of the substrate; this reduces to equation (1) in terms of the symbols used by Edward and Meacock. A convenient method for plotting the data to test this equation by rearranging to equation (3) was noted,^{22a}

$$C_{\text{H}_3\text{O}^+}/k_{\text{eff}} = 1/k + h_0/kK_B \quad (3)$$

and this gave a good linear plot up to the local minimum at *ca.* 75% sulphuric acid. More recently Vinnik and Medvetskaya^{22c} used equation (2) in a slightly modified form (equation 4),

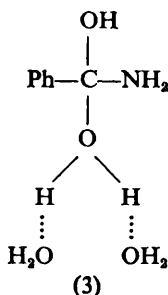
$$k_{\text{eff}} = k C_{\text{H}_3\text{O}^+} f_A^{1-\beta} f_{\text{H}_3\text{O}^+}^{1-\alpha} / (1 + h_0/K_B) \quad (4)$$

where α and β are parameters independent of the solution. They state that for *o*-nitroacetanilide the rate constant is proportional to the hydronium ion concentration, therefore the product $f_A^{1-\beta} f_{\text{H}_3\text{O}^+}^{1-\alpha}$ is independent of water in solution, and the parameters α and β are unity. According to the mechanism proposed, equation (4) operated over the whole sulphuric acid concentration range and the activity of the activated complex was expressed in terms of the product of the ionised form of the anilide and sulphuric acid, $a^* = a_{\text{AH}^+} \cdot a_{\text{H}_2\text{SO}_4}$. This relationship could not be tested because of the lack of physical data for 70–100% w/w sulphuric acid.

The Zucker–Hammett hypothesis⁴⁷ breaks down in even moderately concentrated acids when applied to amides. In 1961 Bunnett¹ presented the Hydration Parameter Treatment, which attempted to overcome the problem of disentangling the roles of proton and water molecule. He plotted $(\log k_{\psi} - \log \alpha)$ *vs.* $\log a_{\text{H}_2\text{O}}$, where α (the fraction of protonated substrate) equals $h_0/(h_0 + K_{\text{AH}^+})$, and obtained slopes (w) which he classified according to mechanism. Yates and Stevens^{45b} criticised Bunnett's relationship, saying that w parameters for reac-

Acidic and Basic Amide Hydrolysis

tions of substrates which do not follow H_0 as bases cannot be simply related to hydration changes in the rate determining step, and devised a modification using H_A to calculate α . Without relying on an acidity function, α can be obtained spectrophotometrically and a value of *ca.* 3 was obtained (over a fairly small acidity range) for the number of water molecules in the transition state (3) during hydrolysis of benzamide.^{45b,49}



The non-linearity of w treatment plots is thought to be due to a decrease in the number of water molecules in the transition state with increasing acid concentration.¹⁷ Bunnett¹ noted that if a w plot showed persistent curvature then a plot of $\log k_\psi - \log [HX]/(h_0 + K_{AH^+})$ vs. $\log a_{H_2O}$ (for a moderately basic substrate) might be linear with a slope of w^* , but this modification did not give linear plots for benzamide, *N*-methylbenzamide, or *NN*-dimethylbenzamide.¹⁷

The linear free energy relationship (l.f.e.r.) proposed by Bunnett and Olsen⁵⁰ is the most general treatment for reaction rates (equation 5)

$$\log k_\psi + H_0 = \phi (H_0 + \log [H^+]) + \log k_2^0 \quad (5)$$

k_2^0 is the second-order rate constant at infinite dilution. The slope parameter ϕ characterises the response of the reaction rate to changing acid conditions and its values are approximately the same for a series of compounds, and may be related to changes in hydration parameters. Bunton *et al.*¹⁷ applied the l.f.e.r. to their data without obtaining linear plots. Bunnett and Olsen⁵⁰ discussed several reasons for non-linearity, including a suggestion that the reaction may be occurring *via* two pathways simultaneously.

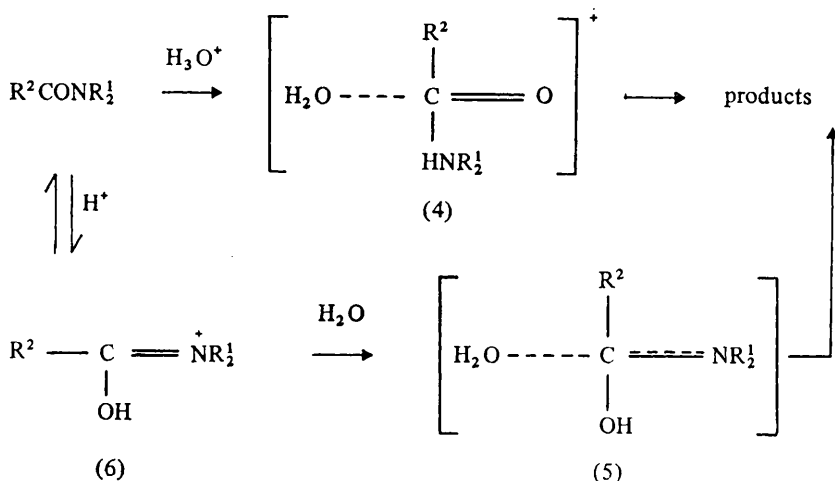
Because all these theories, involving only a one-term mechanistic pathway, seemed to be inadequate for amides, Bunton *et al.*¹⁷ formulated a new reaction scheme involving two transition states (4) and (5), arising from two distinct mechanistic paths involving protonation on both oxygen and nitrogen.

Two assumptions were made, *viz.* the activity coefficients of the free amides.⁵¹ and activity coefficient ratios of ions of like charge¹ are essentially independent of the medium. The Brønsted-Bjerrum rate equation then gives equation (6),

⁴⁹ R. B. Moodie, P. D. Wale, and T. J. Whaite, *J. Chem. Soc.*, 1963, 4273.

⁵⁰ J. F. Bunnett and F. P. Olsen, *Canad. J. Chem.*, 1966, **44**, 1899; 1917.

⁵¹ F. A. Long and W. F. McDevit, *Chem. Rev.*, 1952, **51**, 119; N. C. Deno and C. Perizzolo, *J. Amer. Chem. Soc.*, 1957, **79**, 1345.



Scheme 3

$$k_{\psi} C_A = (k_1 C_A C_{H^+} + k_2 C_A) a_w \quad (6)$$

where C_A is the stoichiometric concentration of amide. Substituting for α (the amount of protonated amide), equation (6) gives equation (7).

$$k_{\psi}/\alpha a_w = k_1 C_{H^+}(1 - \alpha)/\alpha + k_2 \quad (7)$$

This treatment correlated their data well.¹⁷ Criticism of this two-term treatment came from Armstrong, Farlow, and Moodie,⁵² who did not dispute the possibility of both *O*- and *N*-protonation but believed that the assumption about the activity coefficient (*i.e.* cancellation of f_{H^+} with $f^{\dagger}$) was unrealistic. Instead they treated their results for butyramide hydrolysis in sulphuric and perchloric acids by plotting $\log k_p$ vs. $\log a_w$. (k_p is the specific first order rate constant for hydrolysis of the protonated substrate.) The plots showed curvature which they said was typical of a bimolecular mechanism. The hydrolysis data of carbamates has recently been treated similarly.²⁴

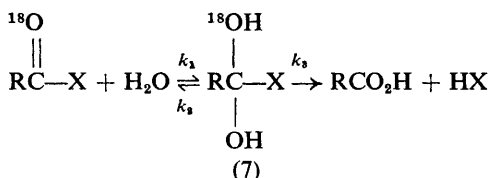
2 Basic Hydrolysis

The kinetics of basic amide hydrolysis are relatively uncomplicated. In 1899 Reid⁶ showed that the reaction was second order, and most recent investigations have included substituent, catalytic, and isotope effects.

A. Mechanism.—The mechanism generally accepted⁶ for basic amide hydrolysis involves a reaction scheme (Scheme 4) in which an intermediate (7) partitions to give products or regenerate reactants, and, provided the oxygen atoms in (7) become equivalent by rapid proton transfers, this reverse step leads to oxygen exchange.

⁵² V. C. Armstrong, D. W. Farlow, and R. B. Moodie, *J. Chem. Soc. (B)*, 1968, 1099.

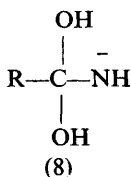
Acidic and Basic Amide Hydrolysis



Scheme 4

The rate constants for exchange and hydrolysis are then related by $k_e/k_h = k_2/2k_3$. In the basic hydrolysis of primary and secondary amides, where $k_e/k_h > 1$, the rate of hydrolysis should therefore be considerably less than that of formation of the intermediate (7).^{29a,30,53}

There is evidence that the equilibration of the oxygen atoms in (7) is not always faster than its breakdown,⁵⁴ and it was suggested that the relatively fast oxygen exchange during the alkaline hydrolysis of amides arose because the negative charge could be located on the nitrogen atom of the intermediate, as in (8).^{30,53}



There is direct evidence for the formation of tetrahedral intermediates in the alkaline hydrolysis of anilides.⁵⁵⁻⁵⁷ The amide residue should be a poorer leaving group than either hydroxy- or alkoxy-groups, and the ready return of (7) to reactants is expected. The alkaline hydrolysis of *NN*-dimethylbenzamide, where structures like (8) cannot be formed, was not accompanied by oxygen exchange.⁵⁸

Bender and Ginger^{29a} found that k_e/k_h for benzamide increased with increasing temperature, and they calculated the activation parameters for the step involving the intermediate and for the difference of two subsequent steps in which the intermediate is partitioned. The ratio decreased with increasing hydroxyl ion concentration and the values were greater in deuterium than in protium oxide. The deuterium solvent isotope effect⁵⁸ upon k_e/k_h suggests either that interoxygen proton transfers are fast or that k_2 and k_3 both depend upon rate-limiting proton transfers between oxygen or nitrogen atoms.

Absence of oxygen exchange during hydrolysis of carboxyl derivatives does

⁵³ M. L. Bender and R. J. Thomas, *J. Amer. Chem. Soc.*, 1961, **83**, 4183; 4189.

⁵⁴ (a) C. A. Bunton and D. N. Spatcher, *J. Chem. Soc.*, 1956, 1079; (b) M. L. Bender, R. D. Ginger, and J. P. Unik, *J. Amer. Chem. Soc.*, 1958, **80**, 1044.

⁵⁵ S. S. Biechler and R. W. Taft, *J. Amer. Chem. Soc.*, 1957, **79**, 4927.

⁵⁶ P. M. Mader, *J. Amer. Chem. Soc.*, 1965, **87**, 3191.

⁵⁷ R. L. Schowen and G. W. Zuorick, *J. Amer. Chem. Soc.*, 1966, **88**, 1223; R. L. Schowen, H. Jayaraman, L. Kershner, and G. W. Zuorick, *ibid.*, 4008.

⁵⁸ C. A. Bunton, B. Nyak, and C. J. O'Connor, *J. Org. Chem.*, 1968, **33**, 572.

not distinguish between unfavourable partitioning of (7) or slow isotopic scrambling of its oxygen atoms. There are many anilide hydrolyses for which proton transfers to and from the leaving group are kinetically important and there is strong evidence that slow proton transfers are involved in the decomposition of tetrahedral intermediates.^{53,57} Bender and Thomas⁵³ explained thus the structural effects upon k_e/k_h for anilide hydrolyses, but Shain and Kirsch⁵⁹ have suggested that this earlier experimental work⁵³ was in error.

The solvent isotope effect, $k_{OH^-}/k_{OD^-} > 1$, for the basic hydrolysis of benzamide⁵⁸ is similar to that found for acetamide,¹⁸ but for *NN*-dimethylbenzamide⁵⁸ the ratio $k_{OH^-}/k_{OD^-} < 1$. This difference was considered to arise because of the solvent isotope effect upon partitioning of (7).

B. Substituent Effects.—Reid⁶ investigated the effect of nuclear substituents on the rates of basic hydrolysis of benzamide and observed that *meta*- and *para*-substituents such as $-NH_2$, $-OMe$, and $-Me$ retarded the rate, whilst $-I$, $-Br$, and $-NO_2$ accelerated it. All *ortho*-substituents retarded it.

Electron-attracting groups lowered the activation energy of substituted-benzamide hydrolyses, while electron-repelling groups raised it.⁸

The structures and reactivities of an extensive range of primary amides, in which substituent changes were confined to alkyl groups, were correlated well by a Taft-type steric energy equation. Bolton^{11b} re-examined these hydrolyses and found the data to be better correlated by an equation including polar, steric, and conjugative influences of the substituents.

Anilides are generally stable towards basic hydrolysis, but compounds containing strongly electron-attracting groups in the acyclic part of the molecule are hydrolysed fairly rapidly. Moreover, the rate may not be proportional to the hydroxide ion concentration. Orders either greater or less than unity have been observed,^{5,29a,53,54a,55-57} when either the amide itself was sufficiently acidic to be partially ionised, *e.g.* trifluoroacetanilide,^{55,56} or when (7) could be further ionised.

C. Catalysis.—Eriksson and Holst⁶⁰ showed that in the hydrolysis of trifluoro- and trichloro-acetanilide, breakdown of (7) to products was catalysed not only by a second hydroxyl ion but also by species able to donate or accept a proton simultaneously, *e.g.* by HCO_3^- , $H_2PO_4^-$, and HPO_4^{2-} ions. Cationic acids of the amine type, *e.g.* monoethanolamine, which cannot both donate and accept a proton in a concerted mechanism and are therefore supposed to act only as general acids, can be very effective catalysts.⁶¹ The reactions were characterised as nucleophilic or general basic attack of the amine and were first-order both in unionised substrate and amine base. Whereas bulky groups completely inhibited the activity of the cation as a general acid, the same groups seemed to assist its

⁵⁹ S. A. Shain and J. F. Kirsch, *J. Amer. Chem. Soc.*, 1968, **90**, 5848.

⁶⁰ S. O. Eriksson and C. Holst, *Acta Chem. Scand.*, 1966, **20**, 1892.

⁶¹ S. O. Eriksson and L. Bratt, *Acta Chem. Scand.*, 1967, **21**, 1812.

Acidic and Basic Amide Hydrolysis

action as a basic catalyst. Thus, *e.g.* triethanolamine was an unexpectedly effective catalyst.

The hydrazinium and hydroxylammonium ions were very effective in catalysing the breakdown of the tetrahedral intermediate formed between trifluoroacetanilide and hydroxyl ions,⁶² and the constants for these catalyses gave, together with the constants for acids of the ammonium cation type,⁶¹ a Brønsted plot with slope 0.9. The reaction rates with these two catalysts were calculated with an equation containing terms first and second order in base.

3 Conclusion

The kinetics of acidic amide hydrolysis are subject to uncertainty. It may be argued that there is no reason why there should be any simple relation between the various parameters. The kinetics of basic amide hydrolysis are, however, clearly understood.

The author thanks Professor Bunton for arousing her interest in this subject, and Gorham Milbank and James Barnett for assistance.

⁶² S. O. Eriksson, *Acta Chem. Scand.*, 1968, **22**, 892.

EXHIBIT 29

Expression of Neutral Endopeptidase (Enkephalinase) in Heterologous COS-1 Cells

CHARACTERIZATION OF THE RECOMBINANT ENZYME AND EVIDENCE FOR A GLUTAMIC ACID RESIDUE AT THE ACTIVE SITE*

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From the ‡Département de Biochimie, Faculté de Médecine, Université de Montréal, Montréal, Canada and the §Département de Chimie Organique, U 266 Institut de la Santé et de la Recherche Médicale, UA 498 Centre National de la Recherche Scientifique, Faculté de Pharmacie, 75006 Paris, France

Neutral endopeptidase (EC 3.4.24.11) is an integral membrane protein found in the plasma membrane of many cell types. The cDNA coding for the complete primary structure of neutral endopeptidase has recently been cloned and sequenced (Devault, A. Lazure, C., Nault, C., Le Moual, H., Seidah, N. G., Chretien, M., Kahn, P., Powell, J., Mallet, J., Beaumont, A., Roques, B. P., Crine, P., and Boileau, G. (1987) *EMBO J.* 6, 1317-1322). Comparison of the sequence of neutral endopeptidase with that of thermolysin, a bacterial Zn-metalloendopeptidase, suggests that Glu-584 in neutral endopeptidase probably corresponds to Glu-143 in thermolysin, which is an essential amino acid involved in catalysis. To test directly the importance of Glu-584 in the catalytic activity of neutral endopeptidase by site-directed mutagenesis, we have constructed an expression vector in which the rabbit kidney cDNA encoding the entire neutral endopeptidase sequence is introduced downstream from the SV40 virus early promoter. After transfection in COS-1 monkey kidney cells, this vector was found to promote the expression of a protein with biochemical and catalytic properties identical to kidney neutral endopeptidase. Oligonucleotide-directed mutagenesis of Glu-584 to either valine or aspartic acid completely abolished the enzymatic activity of the recombinant protein without changing its affinity for the substrate-related tritiated inhibitor [³H]N-[(2*R*,2*S*)-3-hydroxyamino-carbonyl-2-benzyl-1-oxopropyl]-glycine. This observation clearly identifies Glu-584 as one of the important residues responsible for the catalytic activity of the enzyme.

many tissues (Kenny, 1986). Neutral endopeptidase is an ectoenzyme with its active site exposed at the cell surface (Kenny and Maroux, 1982) and hydrolyses peptide bonds involving the amino group of hydrophobic amino acid residues (Matsas *et al.*, 1984). The best substrates for the enzyme appear to be small peptides rather than proteins. It has therefore been suggested that neutral endopeptidase could be important for inactivating small regulatory peptides at the cell surface (Bowes and Kenny, 1986). In mammalian brain, the enzyme has been shown to be involved in the inactivation of the opioid peptides methionine- and leucine-enkephalins (Malfroy *et al.*, 1978; Almenoff *et al.*, 1981) and is therefore often called "enkephalinase."

We have recently deduced the entire amino acid sequence of rabbit neutral endopeptidase from kidney cDNA clones (Devault *et al.*, 1987). Neutral endopeptidase is a 749-residue protein consisting of a short NH₂-terminal cytoplasmic domain (27 residues), a single membrane spanning segment (23 residues), and an extracellular domain that comprises most of the protein mass. This ectodomain also contains 5 asparagine residues that are part of consensus sequences for *N*-glycosylation as well as 12 cysteine residues that may be involved in disulfide bridges stabilizing the conformation of the active enzyme (Tam *et al.*, 1985).

There is very little overall homology between neutral endopeptidase and other Zn-metalloproteases (Devault *et al.*, 1987; Malfroy *et al.*, 1987). However, the substrate specificity of neutral endopeptidase and thermolysin, a bacterial metalloendopeptidase (Matthews *et al.*, 1972), clearly indicates large analogies between the active sites of both enzymes (Kerr and Kenny, 1974; Roques and Fournie-Zaluski, 1986). Indeed, most of the important amino acids present in the active site of thermolysin have been conserved in neutral endopeptidase. These include two of the Zn-coordinating residues, His-583 and His-587 in neutral endopeptidase (which most probably correspond to His-142 and His-146 in thermolysin), and two of the essential amino acids involved in catalysis (Glu-584 and His-637 in neutral endopeptidase, and Glu-143 and His-231 in thermolysin). In both enzymes these amino acids are found within similar sequences (Devault *et al.*, 1987).

In this paper we report the synthesis of recombinant neutral endopeptidase after transfection of monkey kidney cells with an SV40-derived expression vector and the use of this system to test directly the importance of Glu-584 in the catalytic activity of neutral endopeptidase by site-directed mutagenesis. Our results show that the replacement of Glu-584 by

Neutral endopeptidase (EC 3.4.24.11) is a membrane-bound Zn-metalloendopeptidase located in the plasma membrane of

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§ Recipient of a medical Research Council of Canada studentship.

|| Recipient of a Scientist Award from the Medical Research Council of Canada.

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either an aspartic acid or valine residue completely abolishes the catalytic activity of the recombinant enzyme.

EXPERIMENTAL PROCEDURES

Materials—Peptides Tyr-Gly-Gly and Leu-enkephalin were bought from Institut Armand-Frappier (Laval, Québec). The tritiated inhibitor [^3H]N-(2*R*,2*S*)-3-hydroxyaminocarbonyl-2-benzyl-1-oxopropyl-glycine ([^3H]HACBO-Gly, ^{1} 30 Ci/mmol) (Waksman *et al.*, 1985) was synthesized in the laboratory of one of us (B. P. R.) and tritiated at the Commissariat à l'Energie Atomique (CEA, Saclay, France). All the other radiochemicals were bought from Amersham Corp. Phosphoramidon was from Sigma. Thiorphan was synthesized in the laboratory of B. P. R.

Construction of Vector and Transfection of COS-1 Cells—Vector pSVENK19 was constructed from plasmids pENK19 and pSVP3. pENK19 contains the entire neutral endopeptidase cDNA sequence as previously reported (Devault *et al.*, 1987) and pSVP3 is a eukaryotic expression vector using the SV40 early promoter to direct the transcription of pro-opiomelanocortin (POMC) cDNA (Noël *et al.*, 1987). Fig. 1 schematizes the different steps in the construction of pSVENK19. First, pSVP3 was digested with endonuclease *Ava*I, and the ends of the vector were filled in with the Klenow fragment of DNA polymerase I. This digestion removes all POMC coding sequences, leaving 85 nucleotides of POMC mRNA 5' untranslated sequences and 30 nucleotides of the 3' untranslated sequences. Second, pENK19 was digested with endonuclease *Hpa*I to release a DNA fragment of 2598 base pairs containing all of neutral endopeptidase coding sequences, 19 nucleotides of the mRNA 5' untranslated sequences, and 486 nucleotides of the 3' untranslated sequences. Finally, this DNA fragment was ligated to the *Ava*I-cut pSVP3 to generate pSVENK19. The proper orientation of the DNA fragment in pSVENK19 was verified by restriction nuclease mapping. Preparation of the plasmid DNA and transfection of COS-1 cells were as described previously (Noël *et al.*, 1987).

Binding of ^{125}I -Labeled Antibodies to Transfected COS-1 Cells—mAb 2B12 (Crine *et al.*, 1985) was iodinated by the chloramine-T method (Stahli, 1983) to a specific activity of 2×10^7 cpm/ μg . At various times post-transfection, cell monolayers were incubated in phosphate-buffered saline containing 1% bovine serum albumin (PBS-BSA) for 30 min at 37 °C to saturate nonspecific binding sites. The radiolabeled antibody (1×10^6 cpm/150-mm Petri dish) was then added in fresh PBS-BSA and incubation was resumed for 30 min at 37 °C. After several washes in PBS-BSA, cells were lysed in 0.5 M NaOH and the radioactivity measured.

Incubation of Transfected COS-1 Cells with [^3S]Methionine and Immunoprecipitation of Labeled Neutral Endopeptidase—Metabolic labeling of COS-1 cell proteins was performed 40–48 h after transfection. Cells were washed in Tris-buffered saline (TBS) and incubated for 1 h in methionine-free RPMI medium. The incubation medium was then supplemented with [^3S]methionine (60 Ci/mmol; 100 $\mu\text{Ci}/\text{ml}$, Amersham Corp.) and incubation continued for 5 h. Proteins were solubilized in TBS containing 1% octylglucoside, 2 mM EDTA, and aprotinin (16 $\mu\text{g}/\text{ml}$) (buffer A). Immunoprecipitation was performed by incubating cell extracts for 16 h at 4 °C with protein A-agarose (Boehringer-Mannheim, Canada) previously saturated with mAb 2B12. Proteins were eluted in Laemmli's sample buffer (Laemmli, 1970) and electrophoresed on an 8% polyacrylamide gel. Proteins used as standards were: phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (30,000), trypsin inhibitor (20,000), and lactalbumin (14,000).

Electron Microscopy—Colloidal gold particles with a mean diameter of 15 nm were prepared by the method of Frens (1973) using sodium citrate as a reducing agent. The gold particles were complexed to mAbs by standard methods (De Mey *et al.*, 1981; Roth, 1982). Immunogold labeling of cultured cells was usually carried out 40–48 h after transfection. Cell monolayers were first rinsed in PBS and incubated in PBS-BSA for 30 min at 22 °C. Labeling was performed by adding the immunogold reagents resuspended in the same buffer (final $A_{525} = 0.3$) and incubating for an additional period of 30 min

at 22 °C. After labeling, cell monolayers were washed first in PBS-BSA and then in PBS alone. The cells were fixed in PBS containing 3% glutaraldehyde and processed for electron microscopy. For some experiments, cells were first permeabilized by a modification of the method of Willingham (1983) before adding the immunogold reagents. Briefly, cell monolayers were incubated for 30 min at 22 °C in PBS containing 1% saponin, 1% BSA, and 5% sucrose (buffer B). The immunogold reagent in buffer B was then added (final $A_{525} = 0.3$) and the incubation resumed for 30 min. Finally the cells were washed in buffer B without BSA and fixed in PBS containing 5% sucrose and 3% glutaraldehyde.

Enzyme Assays—Neutral endopeptidase was first solubilized from COS-1 cells or kidney brush border membranes (Aubry *et al.*, 1987) in buffer A lacking EDTA. Immunoprecipitation of the enzyme was performed as described (Crine *et al.*, 1985) and the amount of neutral endopeptidase in the immunoprecipitate was determined by a dot-blot assay using mAb 2B11-gold (Moeremans *et al.*, 1984). Known amounts of pure kidney neutral endopeptidase were used to construct a calibration curve. The spots obtained after silver enhancement were quantitated by microdensitometric scanning. The limit of sensitivity of this method is around 1 ng of neutral endopeptidase. Unless otherwise stated, all the enzymatic assays were performed in 0.05 M Tris, pH 7.4 at 25 °C, using [tyrosyl-3,5- ^3H]Leu-enkephalin (25 nM; 34 Ci/nmol, Amersham Corp.) as a substrate. The concentration of the substrate was varied by the isotopic dilution method. Separation of metabolites from the substrate was performed by chromatography on Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA) as described elsewhere (Crine *et al.*, 1985). The K_m values were calculated from Eadie-Hofstee plots.

HPLC Analysis of Leu-enkephalin Metabolites—For assessing the specificity of the enzyme, [tyrosyl-3,5- ^3H]Leu-enkephalin was completely digested with an excess of neutral endopeptidase purified from COS-1 cell extracts. The reaction medium was then analyzed by HPLC on a C_{18} $\mu\text{Bondapak}$ column (Waters). Elution of the metabolites was performed by washing the column successively with the following buffers at a flow rate of 1.5 ml/min: from 0 to 15 min, an isocratic step of 100% of solvent A (0.15 M $\text{Na}_2\text{HPO}_4 \cdot \text{HClO}_4$, pH 2.85, 2.5% methanol); from 15 to 25 min, a linear gradient from 100% solvent A to 52% of solvent A and 48% of solvent B (methanol); from 25 to 40 min, an isocratic step of 52% of solvent A and 48% of solvent B.

Site-directed Mutagenesis—For site-directed mutagenesis, a neutral endopeptidase cDNA fragment (λ ENK7; Devault *et al.*, 1987) was cloned in an M13 vector. The conversion of the Glu-584 codon (GAA) to either Asp (GAC) or Val (GTA) was accomplished by oligonucleotide-directed mutagenesis according to the method of Taylor *et al.* (1985). Mutant cDNAs were screened by sequencing (Sanger *et al.*, 1977), and a *Xba*I-*Bst*EII fragment containing the mutated region was isolated from the replicating form of the M13 recombinant phage and substituted in pSVENK19 to generate either pSVENK19-I (Glu to Val mutation) or pSVENK19-II (Glu to Asp mutation). Both pSVENK19-I and pSVENK19-II were characterized by restriction mapping to ensure that no rearrangement of DNA had occurred and the mutated region sequenced (Maxam and Gilbert, 1977) to confirm that the mutation had been introduced in the expression vector.

Binding of [^3H]HACBO-Gly to COS-1 Cells—Binding was performed on COS-1 cells 40–48 h after transfection. Cells were harvested from Petri dishes by treatment with 4 mM EGTA in TBS. Binding of [^3H]HACBO-Gly was assayed in TBS at 22 °C for 45 min using 4×10^5 cells. Cells were then collected by centrifugation and the pellet was washed twice with cold TBS. Cells were dissolved in Protosol, and radioactivity was quantitated by liquid scintillation counting.

RESULTS

Expression of Neutral Endopeptidase by COS-1 Cells—COS-1 cells were transfected with an SV40-derived expression vector containing the entire coding sequence of neutral endopeptidase inserted downstream from the SV40 early promoter. This vector is called pSVENK19 (Fig. 1). The expression of neutral endopeptidase was monitored with 2B12 monoclonal antibody (mAb) as previously described (Crine *et al.*, 1985). This mAb recognizes a conformational epitope located

¹ The abbreviations used are: [^3H]HACBO-Gly, [^3H]N-(2*R*,2*S*)-3-hydroxyaminocarbonyl-2-benzyl-1-oxopropyl-glycine; POMC, pro-opiomelanocortin; mAb, monoclonal antibody; EGTA, [ethylenedis(oxyethylenenitrilo)]tetraacetic acid; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; BSA, bovine serum albumin; MES, 4-morpholineethanesulfonic acid; HPLC, high pressure liquid chromatography.

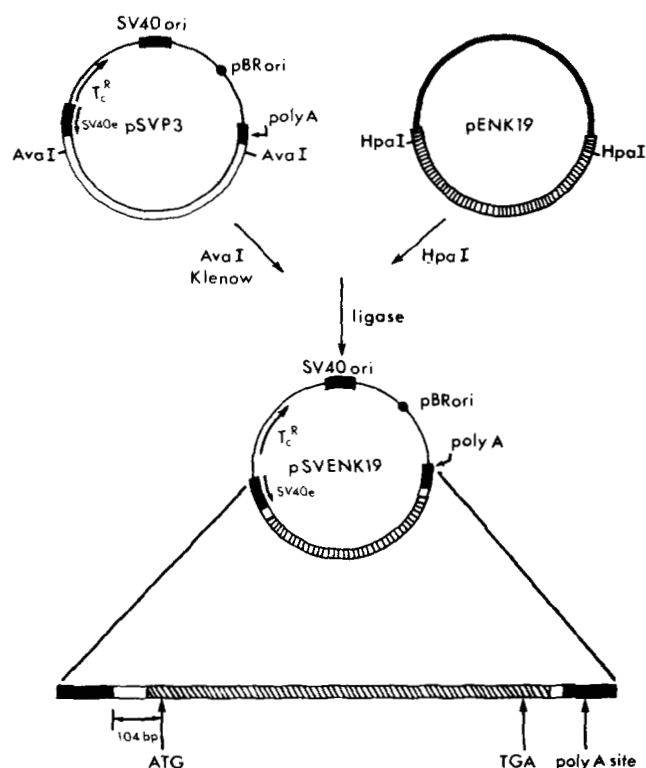


FIG. 1. Construction of vector pSVENK19. Vector pSVENK19 was constructed by inserting a *Hpa*I fragment containing all neutral endopeptidase coding sequences downstream of the SV40 early promoter in plasmid pSVP3 deleted of all POMC coding sequences. (See "Experimental Procedures" for details). Thin lines indicate pBR327 sequences; thick lines, pUC19 sequences; filled boxes, SV40 sequences; open boxes, porcine POMC cDNA; hatched boxes, rabbit kidney neutral endopeptidase cDNA.

on the extracellular domain of neutral endopeptidase.² This antibody was first radioiodinated as described under "Experimental Procedures" and used in a binding assay on intact cells still attached to the culture dishes. As seen in Fig. 2A, COS-1 cells transfected with pSVENK19 started to bind measurable amounts of ¹²⁵I-labeled 2B12 mAb 6 h after transfection. Binding increased thereafter reaching a maximum 40 h after transfection. As a control, COS-1 cells were also transfected with pSVP3 (see Fig. 1), a vector containing the cDNA coding for an unrelated protein (pro-opiomelanocortin). Control cells did not bind any radioactivity (Fig. 2A). These results therefore suggest that pSVENK19 specifically directs the synthesis of rabbit neutral endopeptidase in transfected COS-1 cells. Moreover, since the antibody cannot penetrate the cells, it appears that at least part of the enzyme expressed by transfected COS-1 cells is correctly transported to the cell surface.

Immunoprecipitation of Biosynthetically Labeled Neutral Endopeptidase Expressed in COS-1 Cells—In order to characterize further the immunoreactive material, COS-1 cells were transfected either with pSVENK19 or pSVP3 as a control, and incubated in the presence of [³⁵S]methionine for 5 h. Membrane proteins were solubilized with octylglucoside and immunoprecipitated with 2B12 mAb. Analysis of the immunoprecipitate by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed a protein of 94,000 daltons in the extract of pSVENK19-transfected cells (Fig. 2B, lane 2) which was absent from the extract of control cells (Fig. 2B, lane 1).

This protein band migrated at *M_r* identical to that of the native enzyme purified from rabbit kidneys (Aubry *et al.*, 1987).

The primary structure of neutral endopeptidase predicted from the cDNA sequence contains 5 asparagine residues that are part of the consensus sequence Asn-X-Ser/Thr for N-glycosylation. Digestion of neutral endopeptidase purified from rabbit kidney, with peptide:N-glycosidase F shifts the *M_r*, as measured by its mobility on sodium dodecyl sulfate gels, from 94,000 to 85,000 (Devault *et al.*, 1987). The fact that the enzyme synthesized by COS-1 cells has an apparent molecular weight identical to that of the native kidney enzyme suggests that the enzyme has received a similar amount of glycosylation in this heterologous system.

Orientation of Neutral Endopeptidase in the COS-1 Cell Plasma Membrane—Neutral endopeptidase is a membrane glycoprotein anchored in the lipid bilayer by a 23-residue hydrophobic segment located near its amino terminus. To determine the orientation of neutral endopeptidase in the plasma membrane of COS-1 cells, cell monolayers were transfected with pSVENK19 or pSVP3 and incubated with antibody-gold particle complexes of either 2B12 or 23B11 mAbs. The 2B12 mAb recognizes a conformational epitope on the ectodomain of the enzyme while 23B11 mAb binds a continuous epitope located in its cytoplasmic domain.² After an incubation period of 30 min, the cells were collected and processed for electron microscopy. Fig. 3 clearly shows that when COS-1 cells were transfected with pSVENK19, intense labeling was obtained with 2B12-gold complexes on the extracellular side of the plasma membrane (Panel A). There was no labeling when the cells were transfected with pSVP3 as a control (Panel B) or when labeling was performed with 23B11-gold (not shown). However labeling of pSVENK19-transfected COS-1 cells with 23B11-gold could be obtained by permeabilizing the cells with saponin before incubation with the immunogold reagent. Under those conditions labeling could be observed on the cytoplasmic side of the plasma membrane (Panel C). When the 23B11-gold reagent was used with control cells transfected with pSVP3, no labeling was observed (Panel D). Taken together these results indicate that most of the neutral endopeptidase molecules synthesized by COS-1 cells are transported to the cell surface and correctly inserted in the membrane.

Activity and Specificity of Neutral Endopeptidase Synthesized in COS-1 Cells—Neutral endopeptidase is known to hydrolyze specifically the Gly³-Phe⁴ bond of the opioid pentapeptides methionine- and leucine-enkephalin (Malfroy *et al.*, 1978; Almenoff *et al.*, 1981). Neutral endopeptidase activity in extracts of transfected COS-1 cells was therefore assayed with [³H]leucine-enkephalin as a substrate. Monolayers of cells were transfected with either pSVP3 or pSVENK19 and extracted with octylglucoside 44 h later. Neutral endopeptidase was then immunopurified by adsorption to 2B12 antibody coupled to protein A-Sepharose, and its activity was monitored by measuring the rate of hydrolysis of the Leu-enkephalin substrate. Hydrolysis of the substrate was observed with the extract of cells transfected with pSVENK19 but not with pSVP3 (not shown). Furthermore, the enzymatic activity of the extract could be completely inhibited by addition of 1 μM thiorphan or 1 μM phosphoramidon (not shown), two well characterized neutral endopeptidase inhibitors (Roques *et al.*, 1980; Kerr and Kenny, 1974). The specificity of the enzyme synthesized by pSVENK19-transfected COS-1 cells was also verified by analyzing the peptides resulting from the hydrolysis of [³H]Leu-enkephalin by reversed phase HPLC. As can be seen in Fig. 4, a single radioactive peak

² M. Aubry, C. LeGrimellic, and P. Crine, unpublished results.

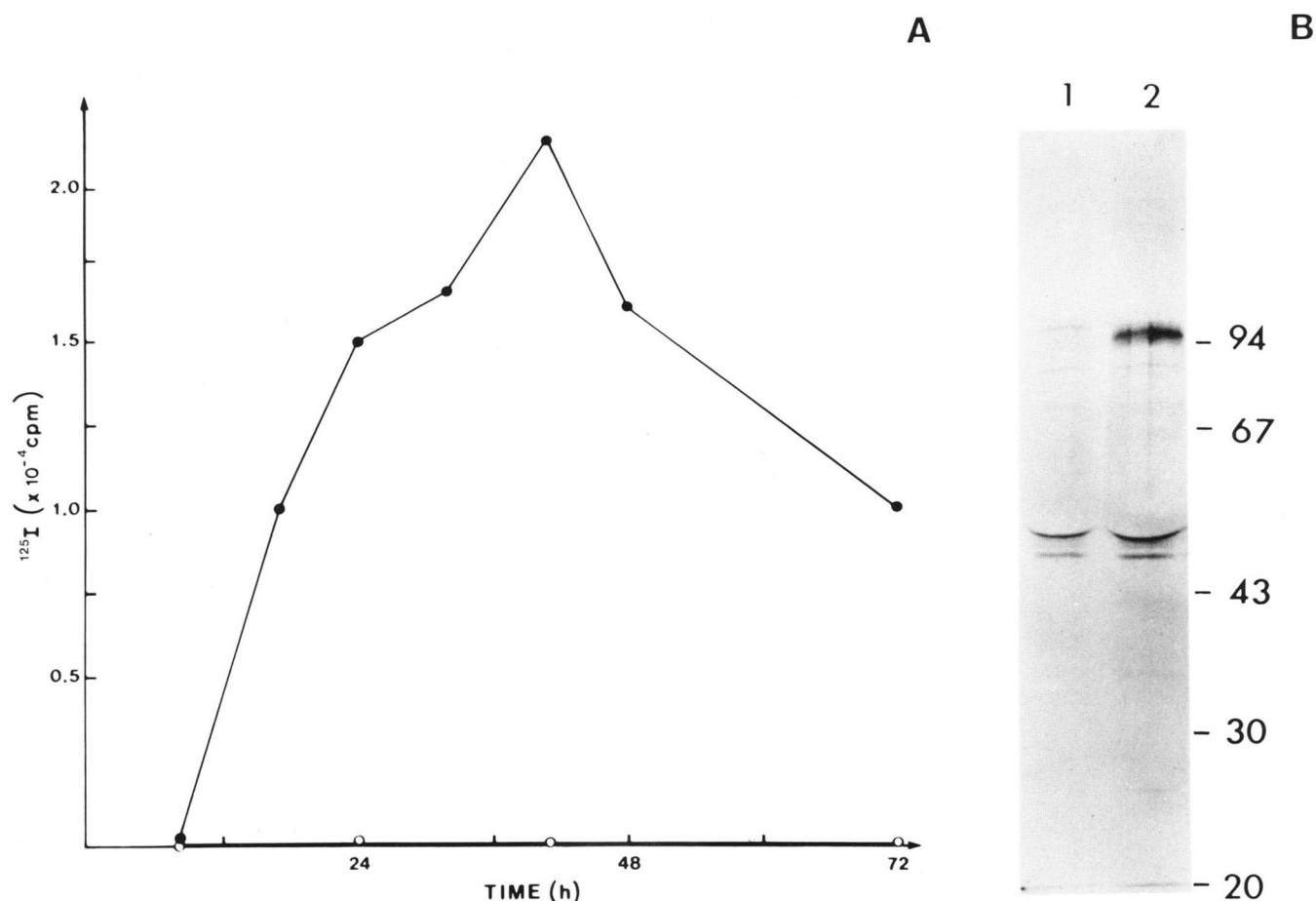


FIG. 2. Expression of neutral endopeptidase in COS-1 cells. A, binding of iodinated 2B12 mAb to transfected COS-1 cells. Binding of 2B12 mAb to monolayers of COS-1 cells transfected with pSVP3 (solid symbols) or pSVENK19 (open symbols) was carried out at different times after transfection. The binding of mAb was assessed by measuring the amount of radioactivity associated to the cell pellet. B, autoradiogram of immunoprecipitated [^{35}S]methionine-labeled proteins from transfected COS-1 cells. COS-1 cells transfected with pSVP3 (lane 1) or pSVENK19 (lane 2) were labeled with [^{35}S]methionine for 5 h. After extraction of the cells with octylglucoside, the proteins were immunoprecipitated with 2B12 mAb and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

comigrating with the tripeptide Tyr-Gly-Gly used as a standard could be detected on the chromatogram, as expected from the known specificity of neutral endopeptidase (Guyon *et al.*, 1979).

Characteristics of Recombinant Rabbit Neutral Endopeptidase—To investigate whether the characteristic properties of the native enzyme are shared by the recombinant gene product, we next compared several typical parameters of the enzyme produced by COS-1 cells with those of its natural homologue purified from rabbit kidneys. We first examined the pH and temperature optima for both enzymes. The pH optimum was determined by progressively increasing the pH of the standard Tris buffer from 7.0 to 9.0 or by replacing it with MES buffers of varying pH values from 5.0 to 7.0. As seen in Fig. 5A, the pH optimum for both the recombinant and native rabbit kidney enzyme is around 7.0. The temperature optimum was determined by performing the standard Leu-enkephalin hydrolysis assay at temperatures ranging between 5 and 55 °C (Fig. 5B). A functional optimum for the 30-min reaction was found at 37 °C for both the recombinant and native kidney enzyme.

We next studied the effect of thiorphan, a potent inhibitor of neutral endopeptidase on the hydrolysis of [^3H]Leu-enkephalin by both the recombinant and native kidney enzymes. Very similar dose-response curves were obtained in both cases

(Fig. 5C). Furthermore, the IC_{50} values of 5.1 and 7.9 nM obtained for the recombinant enzyme and native kidney neutral endopeptidase, respectively, were very close to previously published results (Scott *et al.*, 1985; Fournié-Zaluski *et al.*, 1986). Finally we used Eadie-Hofstee plots to calculate the K_m values of the two enzymes using [^3H]Leu-enkephalin as a substrate (Fig. 5D). A K_m value of 16 μM was found for neutral endopeptidase produced in COS-1 cells, which is very similar to that of 18 μM determined for the enzyme purified from the kidney.

Site-directed Mutagenesis of Neutral Endopeptidase—X-ray diffraction studies have indicated that Glu-143 is involved in the catalytic action of the bacterial Zn-metalloendopeptidase thermolysin. In neutral endopeptidase, Glu-584 is found in a sequence very similar to the segment of thermolysin containing Glu-143 (Fig. 6). To test directly the involvement of Glu-584 in the active site of neutral endopeptidase, conversion of the Glu-584 codon to either Asp or Val codons was accomplished by oligonucleotide-directed mutagenesis of the cDNA. Several Petri dishes of COS-1 cells were transfected with either pSVENK19, pSVENK19-I, or pSVENK19-II to promote the synthesis of normal Glu-584-neutral endopeptidase, Val-584-neutral endopeptidase, and Asp-584-neutral endopeptidase. One dish from each transfection was used to monitor the presence of neutral endopeptidase at the cell surface

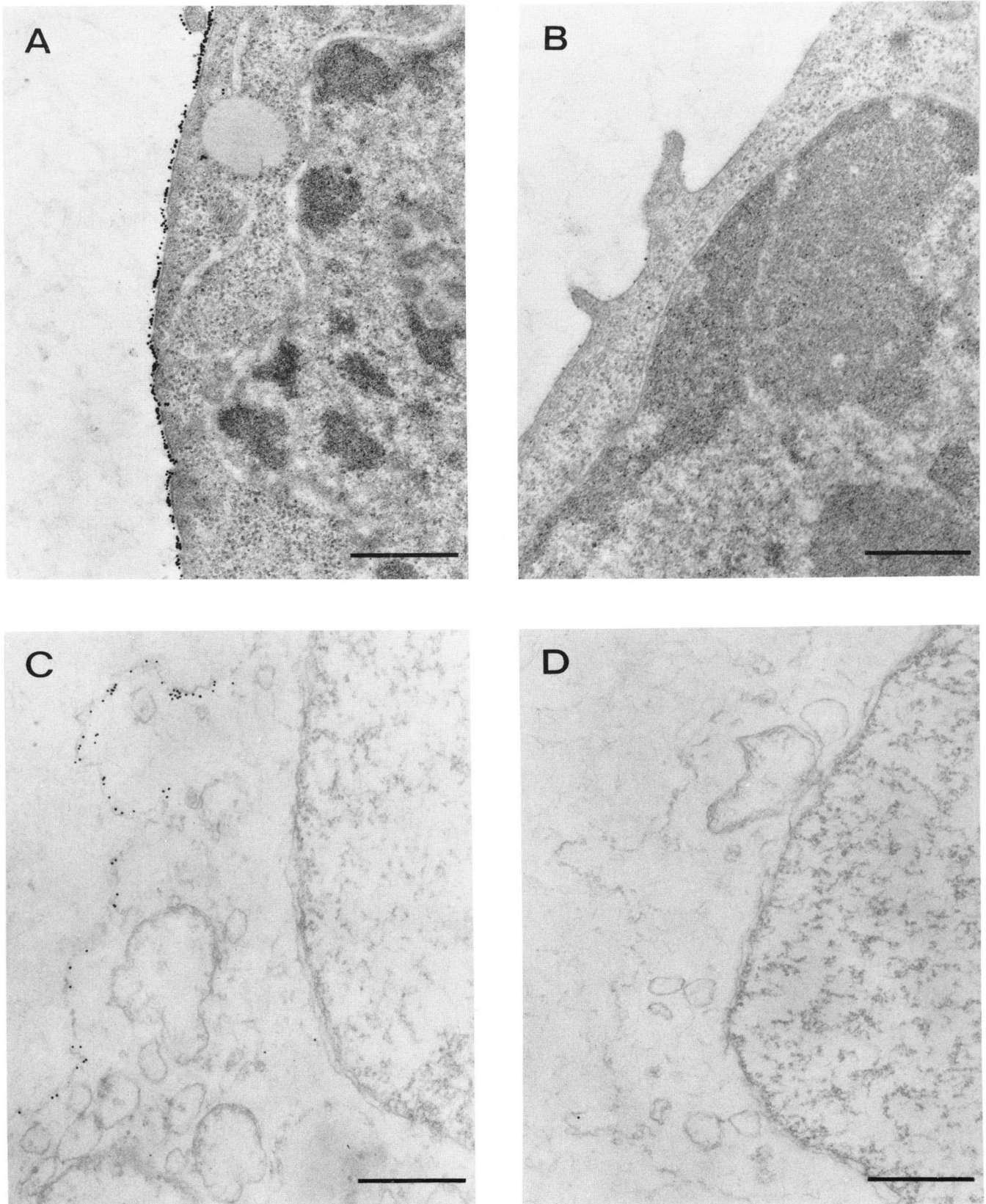


FIG. 3. Electron micrographs of transfected COS-1 cells labeled with gold-antibody complexes. Cells transfected with pSVP3 or pSVENK19 were labeled with 2B12 mAb or 23B11 mAb gold-antibody complexes and examined by electron microscopy. *A*, cells transfected with plasmid pSVENK19 and labeled with mAb 2B12. *B*, cells transfected with plasmid pSVP3 and labeled with mAb 2B12. *C*, cells transfected with plasmid pSVENK19 and permeabilized with saponin before labeling with mAb 23B11. *D*, cells transfected with pSVP3 and permeabilized with saponin before incubation with mAb 23B11.

by binding with the 2B12 mAb. Nearly identical levels of binding were obtained using normal or mutated expression vectors (not shown). Cells from the remaining dishes were collected, and the enzymes were purified from octylglucoside cell extracts as described above. To compare the activity of

mutated neutral endopeptidase with that of Glu-584-neutral endopeptidase, kinetics on the substrate [^3H]Leu-enkephalin were studied. Assays were performed on equal amounts of neutral endopeptidase as determined by immunogold blots (see "Experimental Procedures"). Fig. 7a shows that Glu-584-neutral endopeptidase hydrolyzed approximately 80% of the substrate present in the assay over a period of 30 min. During the same period, no activity could be detected for either Asp-584-neutral endopeptidase or Val-584-neutral endopeptidase. Increasing the amount of mutated enzyme in the assay by a factor of 6 did not allow the detection of enzymatic activity. Since the enzymatic assay can easily detect the hydrolysis of 5 fmol of substrate, we estimate that the activity of the mutated enzymes, as measured by the initial rate of hydrolysis at a substrate concentration of 25 nM, is less than 0.25% of that of Glu-584-neutral endopeptidase. Such a drastic effect could either be due to the inability of the substrate to bind to the mutated enzymes, or to the incapacity of the mutated enzymes to cleave the peptide bond after formation of the Michaelis complex. In order to distinguish between these two possibilities, we next performed binding experiments using the high affinity nonhydrolyzable neutral endopeptidase substrate-derived inhibitor [^3H]HACBO-Gly (Waksman *et al.*, 1985). Binding was assayed directly on transfected cell suspensions. Nonspecific binding was determined in the presence of 1 μM thiorphan and was always less than 10% of the total binding observed. Similar background values were obtained

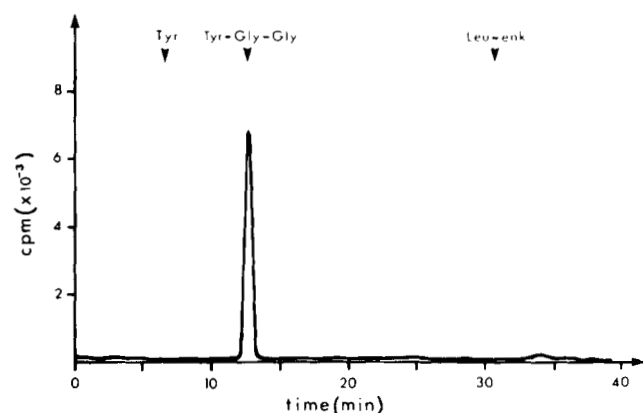


FIG. 4. HPLC analysis of tritiated metabolites of [^3H]Leu-enkephalin. COS-1 cells transfected with pSVENK19 were extracted with octylglucoside and the proteins precipitated with 2B12 mAb. To determine the specificity of the [^3H]Leu-enkephalin degrading activity present in the immunoprecipitated proteins, the tritiated metabolites were analyzed by HPLC. Arrows indicate the elution position of standard peptides.

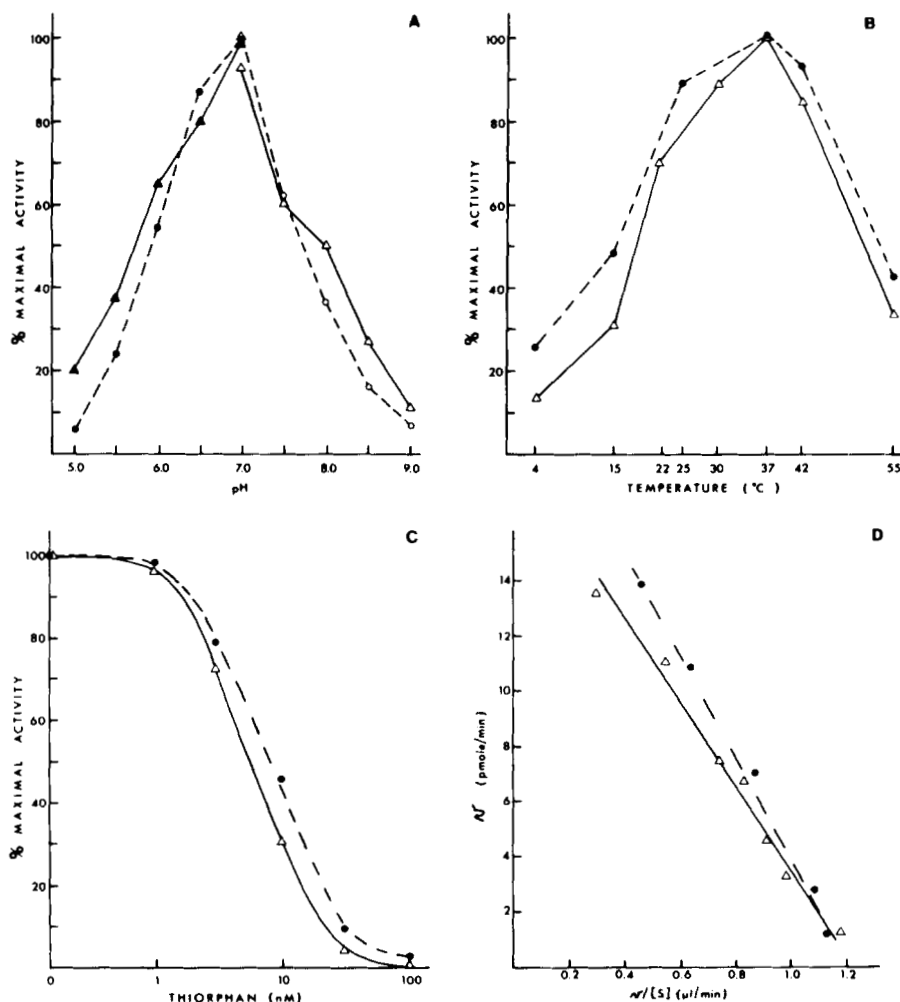


FIG. 5. Characterization of neutral endopeptidase activity produced by COS-1 cells. The pH and temperature optima, the inhibition by thiorphan, and the K_m value for Leu-enkephalin were determined for neutral endopeptidase extracted from COS-1 cells transfected with pSVENK19 and compared to the values found for neutral endopeptidase extracted from rabbit kidney. A, activity as a function of pH. 0.05 M MES (filled symbols) and 0.05 M Tris (open symbols) were used as buffers for the pH ranges of 5.0–7.0 and 7.0–9.0, respectively. B, activity as a function of temperature. C, inhibition of activity by thiorphan. Activity was determined for a substrate concentration of 25 nM in the presence of varying concentrations of thiorphan. D, Eadie-Hofstee plots. Solid symbols, COS-1-produced neutral endopeptidase; open symbols, kidney-produced neutral endopeptidase. See "Experimental Procedures" for details. All assays were performed in triplicate.

Thermo	G	G	I	-	D	V	V	A	H	E	L	T	H	146
NEP	G	G	I	G	M	V	I	C	H	E	I	T	H	587

FIG. 6. Sequence comparison between segments of thermolysin (Thermo) and neutral endopeptidase (NEP). Numbers refer to the last amino acid position in each protein segment. Asterisks indicate the position of Zn-coordinating and catalytic residues in thermolysin. Identical residues are boxed, while conservative amino acid changes are indicated by broken lines. Gaps are represented by dashes.

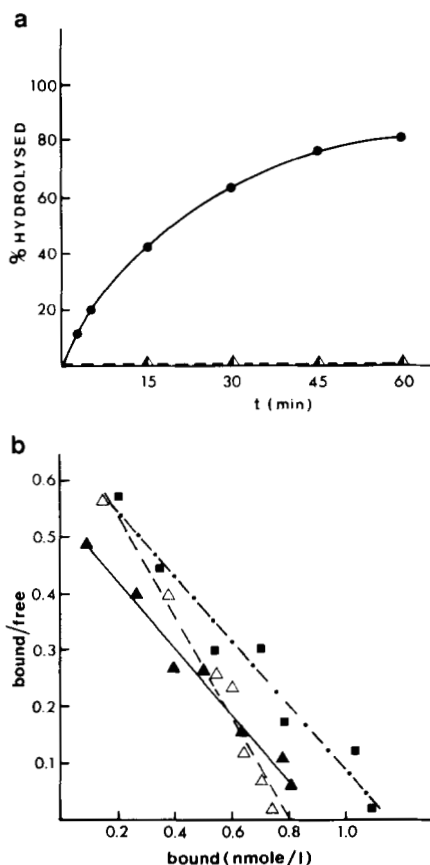


FIG. 7. Enzymatic characterization of normal and mutated neutral endopeptidase. *a*, neutral endopeptidase kinetics of [^{3} H]Leu-enkephalin hydrolysis. COS-1 cells were transfected with either pSVENK19, pSVENK19-I, or pSVENK19-II. Recombinant neutral endopeptidase was immunoprecipitated using 2B12 mAb fixed to protein A-agarose and the activity of normal and mutated neutral endopeptidase was determined by measuring the rate of hydrolysis of [^{3} H]Leu-enkephalin. Filled circles, Glu-584-neutral endopeptidase; open triangles, Asp-584-neutral endopeptidase; filled triangles, Val-584-neutral endopeptidase. All assays were performed in triplicate. *b*, binding of [^{3} H]HACBO-Gly to normal and mutated neutral endopeptidase. Binding was performed on COS-1 cells transfected with either pSVENK19, pSVENK19-I, or pSVENK19-II as described under "Experimental Procedures." Scatchard plots were obtained from specific binding values (performed in triplicate) for Glu-584-neutral endopeptidase (—), Val-584-neutral endopeptidase (—), and Asp-584-neutral endopeptidase (—). K_d values were deduced from the slopes and calculated from two independent experiments: Glu-584-neutral endopeptidase, 1.4 nM; Val-584-neutral endopeptidase, 0.8 nM; Asp-584-neutral endopeptidase, 1.8 nM.

with control cells transfected with the pSVP3 vector (results not shown). The affinity constants for the binding of [^{3} H]HACBO-Gly to enzymes has been determined from Scatchard plots. As shown in Fig. 7b, very similar K_d values were obtained for the normal and the mutated enzymes (1.4, 0.8, and 1.8 nM for Glu-584-neutral endopeptidase, Val-584-neutral

endopeptidase, and Asp-584-neutral endopeptidase, respectively). These results indicate that substitution of either Val or Asp for Glu at position 584 does not interfere with the binding of [^{3} H]HACBO-Gly to the enzyme.

DISCUSSION

The experiments described here were designed to test the importance of selected amino acid residues in the catalytic activity of neutral endopeptidase by site-directed mutagenesis. As a prerequisite, it was essential first to establish and validate a system capable of expressing neutral endopeptidase activity in heterologous cells. From the inserts initially isolated and cloned from kidney cDNA banks, we constructed an expression vector in which the entire coding sequence of neutral endopeptidase was placed under the control of the SV40 early transcription promoter. The vector also contains the origin of replication of the SV40 virus, allowing autonomous replication of the shuttle vector after transfection into COS-1 cells (Gluzman, 1981). Transfected COS-1 cells were found to synthesize substantial amounts of neutral endopeptidase. Furthermore, the enzyme appears to be transported to the cell surface. The insertion of neutral endopeptidase in the plasma membrane was characterized with two monoclonal antibodies, 2B12 and 23B11, which recognize the extracellular and cytoplasmic domains of neutral endopeptidase, respectively. Antibody-gold complexes were found associated with COS-1 cell plasma membrane in a pattern similar to the one previously found in the kidney cortex brush border membrane labeled with the same immunogold reagents.² This is in good agreement with the analysis of the neutral endopeptidase hydropathy plot which predicts that the protein is anchored in the plasma membrane by a 23-residue peptide located near its NH₂ terminus (Devault *et al.*, 1987). It therefore appears that neutral endopeptidase contains, in its structure, all the information required for its proper integration into the plasma membrane and that COS-1 cells can use this information for the correct intracellular transport of the protein.

Our results further demonstrate that the recombinant enzyme encoded by pSVENK19 exhibits biochemical and kinetic properties similar to those of native neutral endopeptidase purified from kidney BBM extracts. The significant similarities include the following. 1) The values for the apparent K_m of the hydrolysis of Leu-enkephalin are similar. 2) Recombinant rabbit neutral endopeptidase, like its natural homologue from the rabbit kidney, is inhibited by thiorphan and phosphoramidon. Furthermore, the IC₅₀ of thiorphan for the recombinant enzyme is very close to the value found in the same conditions for the kidney enzyme. 3) The optimal conditions for the hydrolysis of Leu-enkephalin (pH, temperature) are very similar. 4) From the apparent molecular weight of the recombinant enzyme it appears that COS-1 cells are capable of adding a similar complement of asparagine-linked oligosaccharide side chains to the recombinant enzyme. Thus transfection of an SV40-derived neutral endopeptidase expression vector in COS-1 cells constitutes a very convenient experimental system for undertaking site-directed mutagenesis studies.

Our first target in mutation experiments was Glu-584. This residue is found within a region of neutral endopeptidase that is very similar to the region of thermolysin known to contain many of the amino acids involved in zinc coordination and catalytic activity (Kester and Matthews, 1977; Weaver *et al.*, 1977). It is therefore most likely that Glu-584 of neutral endopeptidase plays a role similar to that of Glu-143 in thermolysin, which is thought to be involved in several steps of peptide bond hydrolysis (Hangauer *et al.*, 1984). To test

this assumption, this Glu was replaced by 2 different residues: valine, a noncharged residue, and aspartic acid, which maintains a negative charge at the same position but retracts the carboxylic group by a distance of approximately 1.4 Å due to shortening of the side chain. Expression of cDNAs harboring these mutations produced enzymes whose activities were reduced at least 400-fold. This effect could result from 1) the change of a key catalyst essential in peptide bond cleavage, 2) change of a crucial residue involved in substrate binding, or 3) the indirect alteration of the active site tertiary structure. Our finding that normal and mutated enzymes showed virtually identical affinities for HACBO-Gly, a substrate-derived neutral endopeptidase competitive inhibitor (Waksman *et al.*, 1985) strongly suggests that Glu-584 is directly involved in peptide bond cleavage.

According to Hangauer *et al.* (1984), Glu-143 of thermolysin plays a major role in catalysis. First, as a base catalyst, it triggers the nucleophilic attack of a water molecule on the carbonyl group of the scissile peptide bond. It also acts as a proton shuttle between the water molecule and the nitrogen of the amide bond to be cleaved. The observation that recombinant neutral endopeptidase enzymes containing mutations at Glu-584 are virtually devoid of activity strongly suggests a similar role for this residue.

Several studies indicate that neutral endopeptidase is a key enzyme in the homeostasis of the opioid peptides methionine- and leucine-enkephalin in mammalian brain (Malfroy *et al.*, 1978; Almenoff *et al.*, 1981). Inhibitors of neutral endopeptidase such as thiorphan (Roques *et al.*, 1980) and retrothiorphan (Roques *et al.*, 1983) are capable of inducing a naloxone-reversible analgesic response. Inhibitors of neutral endopeptidase therefore could represent a new class of analgesic drugs (Dickenson, 1986; Roques and Fournié-Zaluski, 1986). Up to now, the design of these compounds has been based on a generalized active site model for Zn-metalloproteases (Ondetti *et al.*, 1977). Better knowledge of the main structural and functional features of the enzyme would be of great interest for the design of new drugs for clinical applications. Such information could be obtained by performing site-directed mutations on the cDNA and studying the catalytic properties of the mutated enzyme expressed in heterologous cells. It is clear that the system we have established will be very useful for further probing the importance of selected amino acids in the catalytic activity and substrate binding of neutral endopeptidase.

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EXHIBIT 30

COMMUNICATION

Structure of Human Neutral Endopeptidase (Neprilysin) Complexed with Phosphoramidon

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Neutral endopeptidase is a mammalian type II integral membrane zinc-containing endopeptidase, which degrades and inactivates a number of bioactive peptides. The range of substrates cleaved by neutral endopeptidase *in vitro* includes the enkephalins, substance P, endothelin, bradykinin and atrial natriuretic factor. Due to the physiological importance of neutral endopeptidase in the modulation of nociceptive and pressor responses there is considerable interest in inhibitors of this enzyme as novel analgesics and anti-hypertensive agents. Here we describe the crystal structure of the extracellular domain (residues 52–749) of human NEP complexed with the generic metalloproteinase inhibitor phosphoramidon at 2.1 Å resolution. The structure reveals two multiply connected folding domains which embrace a large central cavity containing the active site. The inhibitor is bound to one side of this cavity and its binding mode provides a detailed understanding of the ligand-binding and specificity determinants.

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Keywords: enkephalinase; metalloprotease; phosphoramidon; X-ray structure; endothelin converting enzyme

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Neutral endopeptidase (NEP) (EC 3.4.24.11), also known as neprilysin, enkephalinase, NEP, CALLA or CD10, is a membrane-bound zinc-dependent endopeptidase originally extracted and purified from the brush border of rabbit kidney (Kerr & Kenny, 1974). This ectoenzyme is widely distributed in mammalian tissues and is involved in the inactivation of a variety of signaling peptides (Erdos & Skidgel, 1989; Roques *et al.*, 1993; Turner & Tanzawa, 1997). NEP has been implicated in the regulation of opioid peptide action through the degradation of endogenously released enkephalins (Roques *et al.*, 1980). Furthermore, it is involved in the physiological degradation of the peptides modulating blood pressure, such as the cardiac hormone atrial natriuretic peptide (ANP), bradykinin and endothelin. There is great interest in the identification of novel inhibitors of NEP for application

in clinical treatment (Abassi *et al.*, 1992; Kenny & Stephenson, 1988; Pham *et al.*, 1993). Potent inhibitors of NEP have been synthesized which produce a pharmacological response through increase in opioid or vasoactive peptide levels, indicating their therapeutic potential as novel analgesics or antihypertensive agents (Burnett, 1999; Chen *et al.*, 1998; Robl *et al.*, 1997, 1999; Roques & Beaumont, 1990).

The primary sequence of NEP has been determined by cloning its cDNA from several species (Devault *et al.*, 1987; Malfroy *et al.*, 1987, 1988). NEP is the prototype of a group of metalloproteinases which includes the endothelin-converting enzymes (ECE-1 and ECE-2), the erythrocyte surface antigen KELL, and the PEX gene product. They belong to the M13 subfamily of mammalian neutral endopeptidases and consist of a short N-terminal cytoplasmic domain, followed by a single transmembrane helix, and a large C-terminal extracellular domain that contains the active site (The HYP consortium, 1995; Emoto & Yanagisawa, 1995; Lee *et al.*, 1991; Shimada *et al.*, 1994). The extracellular domain of NEP contains 12 cysteine residues, all involved in disulfide bridges, ten of which are conserved in ECE,

Abbreviations used: NEP, neutral endopeptidase; ANP, atrial natriuretic peptide; TLN, thermolysin; SNEP, soluble extracellular domain of human neutral endopeptidase; NAG, N-acetyl-glucosamine.

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KELL, and PEX (Figure 1). The overall homology between NEP and ECE-1, ECE-2, KELL and PEX is 35 %, 30 %, 31 %, and 25 %, respectively, but increases to 51 %, 44 %, 43 %, and 39 % if only the 250 C-terminal residues are considered. This degree of similarity is sufficient to indicate that these proteins share a common origin and a similar fold.

The catalytic properties of NEP resemble those of a group of zinc-dependent bacterial endopeptidases of which thermolysin (TLN) is the best characterized. Early structure-activity studies showed that TLN and NEP cleave the peptide

bond of their substrates at the amino side of hydrophobic residues and that both enzymes are inhibited by the same type of molecules, such as phosphoramidon and thiorphan, with the same stereochemical dependence (Benchetrit *et al.*, 1987; Fulcher *et al.*, 1982; Hersh & Morihara, 1986). Although there is very limited homology between the primary sequences of mammalian and bacterial enzymes, they are characterized by two consensus sequences HExxH and ExxxD, involved in zinc binding and catalysis (Matthews, 1988). Hydrophobic cluster analysis suggested important similarities in their active sites and putative residues

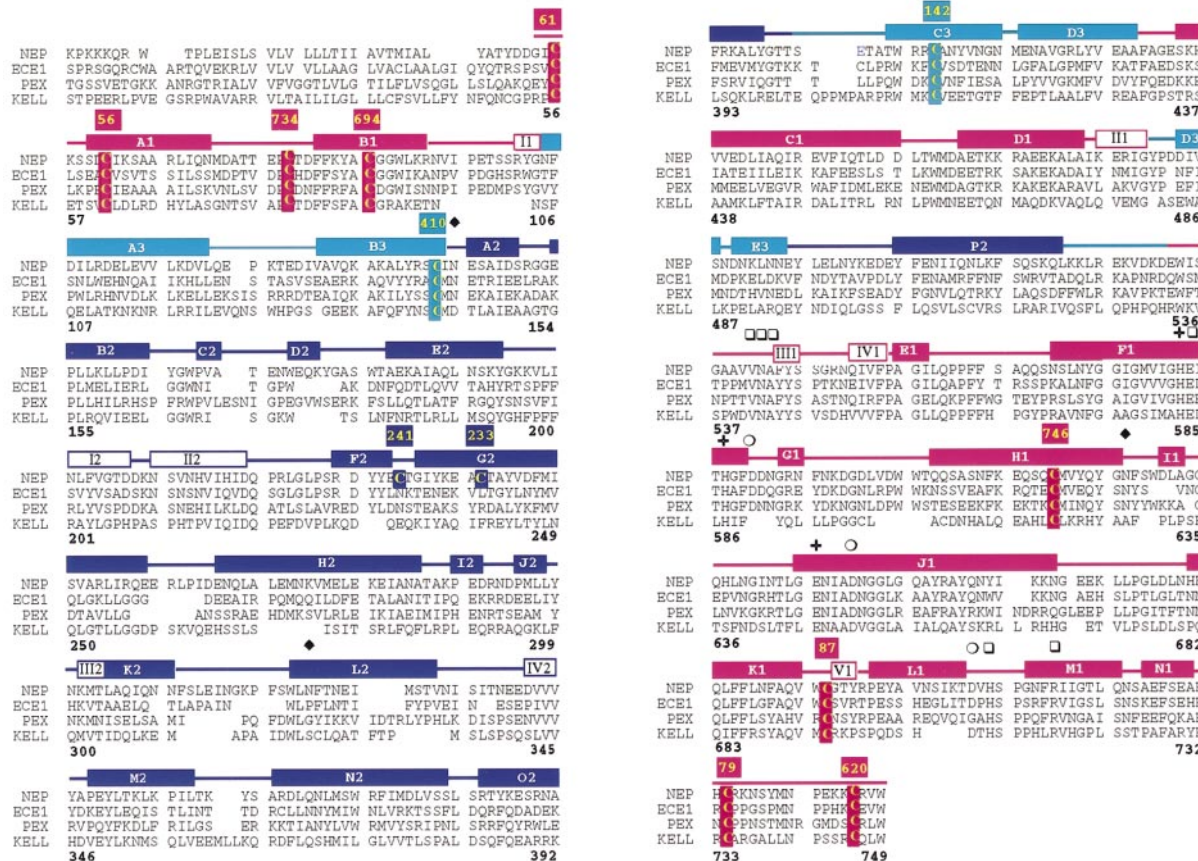


Figure 1. Multiple sequence alignment of selected mammalian membrane metalloproteases NEP, ECE, KELL and PEX. The α -helices, filled cylinders, β -strands, open cylinders, are drawn above the sequence. Coloring as indicated in Figure 2. Zinc-binding residues, crosses; ligand-binding residues; open squares. Residues implicated in the stabilization of the active site are labeled by circles and glycosylation sites are indicated with diamonds. Cysteine residues and their respective disulfide-bond partners are indicated. Protein expression, purification and crystallization will be described in detail elsewhere (Dale *et al.*, unpublished results). Briefly, the extracellular domain of human NEP (residues 52-749) was cloned into pPICZ α -C to create a fusion with the α -mating factor signal sequence and protein produced in the yeast *Pichia pastoris* GS115. Protein expression was accomplished using YP medium with 3 % methanol. The secretion level of sNEP was modest at 10 mg/l. The sNEP could be purified to homogeneity using five chromatographic steps. The N-terminal sequence of purified sNEP indicated that the protein was efficiently processed by the STE13 signal peptidase. The kinetic properties of sNEP were similar to that of the native enzyme (data not shown). Mass spectral analysis of the purified protein resulted in a mass of 92,186 Da (predicted molecular mass is 80,086 Da) indicating that the protein is significantly glycosylated. Treatment of sNEP with Endo-F1 glycosidase resulted in a decrease in approximately 10 kDa giving a final mass of 81,369 Da indicating that a small degree of glycosylation remained. Light-scattering analysis of both sNEP and the de-glycosylated sNEP showed a narrow unimodal size distribution suggesting that they were suitable for crystallization trials.

involved in zinc coordination, catalysis and substrate binding have been identified in NEP by site-directed mutagenesis experiments (Benchetrit *et al.*, 1988; Devault *et al.*, 1988a,b; Dion *et al.*, 1993, 1995; Le Moual *et al.*, 1991, 1994). In the absence of structural information for any member of the neprilysin family, the observed three-dimensional fold for the TLN family has been used to generate a model of the active site region of NEP for the design of new NEP inhibitors (Gaucher *et al.*, 1999; Ksander *et al.*, 1997a,b; Tiraboschi *et al.*, 1999).

In order to aid in the design of potent and selective inhibitors of this structural class, we have solved the three-dimensional structure of human NEP by X-ray crystallography. The soluble extracellular domain (D52 to W749) of human NEP (sNEP) was expressed in the yeast *Pichia pastoris* and the protein purified to homogeneity (Figure 1). Crystals could be obtained for the Endo-F1 glycosidase treated enzyme in the presence of the inhibitor phosphoramidon (*N*-(α -L-rhamnopyranosyl-oxyhydroxy-phosphinyl)-L-leucyl-L-tryptophan), a metabolite produced by *Streptomyces tanashiensis*. The structure was solved by the multiple isomorphous replacement method and refined to a crystallographic *R*-factor of 19.5% (*R*_{free} = 24.2%) at 2.1 Å resolution (Figure 2). Data collection and refinement statistics are summarized in Table 1.

The overall shape of the monomeric ectodomain is an ellipsoid with a major axis of 95 Å and minor axes of approximately 55 Å. It is composed of two largely α -helical domains which can be considered as two lobes connected by intertwining polypeptide segments. A cartoon depicting the overall fold of human sNEP together with the secondary structure assignment is shown in Figure 3. The larger N-terminal domain (domain 1), which follows the trans-membrane anchor of the mature enzyme is 321 residues long and consists of three non-consecutive polypeptide segments (Figure 3). The residues essential for zinc coordination and catalysis are located within this domain. It is structurally

most closely related to other zinc metalloproteases such as TLN, bacterial metallo-elastase and the neutral protease from *Bacillus cereus*. The smaller second domain (domain 2) is 286 residues long and composed of two non-consecutive polypeptide segments and shows a weak structural similarity with glycosyl hydrolase (Tews *et al.*, 1996). The enzyme has six disulfide bridges, five of which are conserved among the other members of the mammalian membrane metallopeptidase family (Figure 1). Four of these are located within the catalytic domain 1, a single disulfide bridge is found within the inter-domain linker fragments, and the non-conserved disulfide bond is present within domain 2. Unlike ECE, which exists as a disulfide-linked homodimer, sNEP is a monomer in solution. However, when E403, located on the surface in the linker region, is mutated to a cysteine residue (C412 in ECE) it results in the formation of a disulfide-bridged homodimer (Hoang *et al.*, 1997). *N*-Glycosylation on N144, N304 and N627 is confirmed by electron density consistent with a single *N*-acetyl-glucosamine (NAG) moiety.

Together with four inter-domain linker fragments both domains form a central, roughly spherical cavity, which bears the active site of the enzyme (Figures 3(a) and 4). The cavity has a diameter of approximately 20 Å and is accessible *via* a small, circular opening formed primarily by charged residues. One of the main differences between the mammalian metallopeptidases of the neprilysin family and the bacterial proteases is that the majority of the natural substrates of NEP are restricted to about 3000 Da. This selectivity is possibly due to the presence of domain 2 (not present in TLN), which restricts the active site access and thus functions as a molecular sieve. The inhibitor phosphoramidon is nestled in the interior of the cavity and binds near the highly conserved residues of the consensus sequences ⁵⁸³HExxH⁶⁸⁷ and ⁶⁴⁶ExxxD⁶⁵⁰ and the residues of the ⁵⁴²NAFY⁵⁴⁵ motif (Figures 1 and 5). The residues belong to the zinc-binding helix F1, the helix J1 forming the

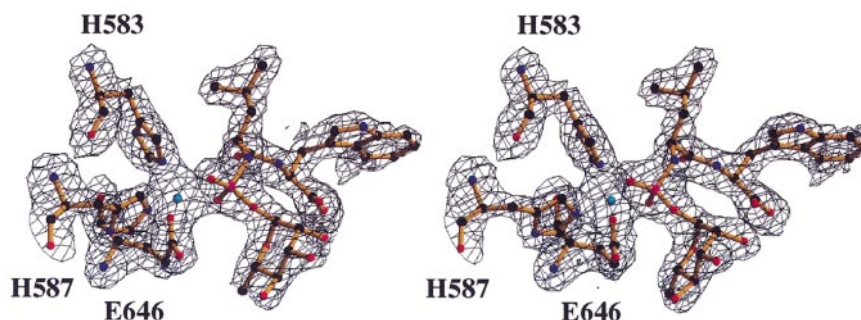


Figure 2. An $F_o - F_c$ omit electron-density map of phosphoramidon, zinc and the three metal binding ligands of the protein (H583, H586 and E646), calculated with phases from the refined model at 2.1 Å resolution. The map is contoured at 3 σ .

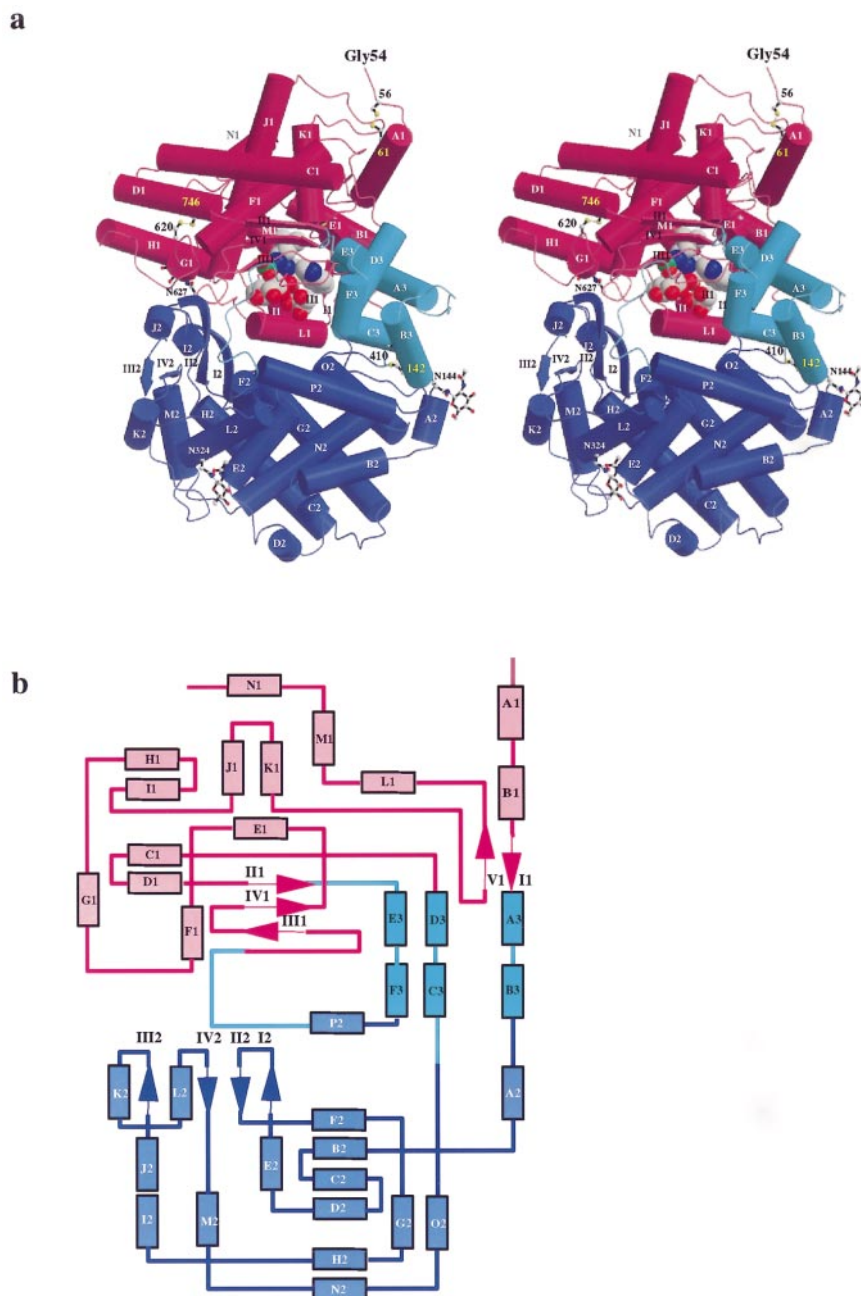


Figure 3. Ribbon plot and topology of sNEP complexed with phosphoramidon. (a) Stereoview of a cartoon model of human neprilysin, showing the catalytic domain 1, violet; the smaller domain 2, blue; and the three inter-domain linker fragments, cyan. The transition-state mimetic phosphoramidon is shown in CPK representation. Glycosylation sites and disulfide bonds are indicated. (b) Topological diagram of sNEP colored as above. Arrows denote strands and cylinders indicate helices numbered as in Figure 1.

base of the active site cleft, and β -strand III1 which acts as a cap holding the well-ordered inhibitor in the active site (Figure 5) (Sansom *et al.*, 1998).

The zinc ion is coordinated in an approximately tetrahedral geometry, as observed in TLN. Its binding involves a single oxygen atom (O1) of the tetrahedral N-phosphoryl moiety of the inhibitor with an inter-atomic distance of 1.93 Å, as well as three ligands from the protein, H583 (2.03 Å), H587 (2.01 Å), and E646 (1.96 Å)

(Figure 5). The side-chains of D590 and D650 appear to be part of two Asp-His-Zinc triads, which involve H587 and H583 on the N and C-terminal site of the N-phosphoryl moiety, respectively. The latter triad is believed to be involved in the precise positioning of the zinc ion for catalysis (Le Moual *et al.*, 1994). A similar role can be expected for the N-terminal triad, which is conserved in all members of the neprilysin family. The side-chain of H711 is hydrogen bonded to the zinc ligand of the inhibitor and

Table 1. Crystallographic data and refinement statistics

Data sets (soaking conditions)	Native-1	Native-2	EuCl ₃ (3 mM, 28 hours)	Thiomersal (5 mM, 120 hours)	K ₃ UO ₂ F ₅ (2.5 mM, 144 hours)
Data collection					
Exposure time/frame (s)	900	120	500	600	600
Angular increment per frame (deg.)	0.5	1.0	0.5	0.5	0.5
Total rotation range (deg.)	75	61	41.5	58.5	55
Crystal to detector distance (mm)	180	360	200	200	200
Unit cell parameters <i>a</i> and <i>c</i> (Å)	107.6, 112.8	107.6, 112.8	107.8, 112.5	107.8, 112.8	107.7, 112.8
Data reduction					
Maximum resolution (Å)	2.44	2.00	2.44	2.44	2.44
Number of measurements	91.966	189.020	65.138	82.229	65.005
Number of unique reflections	26.702	49.828	26.578	26.761	25.817
Completeness (%) ^a	93.7 (79.9) ^a	99.4 (98.9) ^a	93.5 (92.3) ^a	93.5 (95.7) ^a	90.7 (95.5) ^a
<i>R</i> _{sym} ^b	9.6 (37.4)	5.3 (30.1)	11.5 (36.5)	6.6 (20.8)	8.21(37.5)
Heavy-atom refinement parameters					
Figure of merit (acentric/centric)	0.38/0.42				
<i>R</i> _{cullis} ^c (acentric/centric)			0.84/0.48	0.75/0.78	0.89/0.88
Phasing power ^d (acentric/centric)			1.05/0.86	1.07/0.76	0.97/0.79
Refinement statistics					
Resolution range (Å)	20–2.1				
<i>R</i> _{cryst} (<i>R</i> _{free}) (%) ^e	19.5 (24.2)				
Number of protein atoms ^f (mean <i>B</i> in Å ²)	5595 (29.1)				
Number of water molecules	465				
Number of ligand atoms (mean <i>B</i> in Å ²)	37 (20.8)				
Number of glycerol atoms (mean <i>B</i> in Å ²)	6 (44.9)				
Number of NAG atoms (mean <i>B</i> in Å ²)	42 (48.9)				
rmsd ^g bonds (Å)	0.009				
rmsd angles (deg.)	1.48				

^a Values in parentheses are statistics for highest resolution bin.

^b $R_{\text{sym}} = \sum_i \sum_j |I_i(h) - \langle I(h) \rangle| / \sum_i \sum_j I_i(h)$, where $I_i(h)$ and $\langle I(h) \rangle$ are the *i*th and mean measurement of the intensity of reflection *h*.

^c $R_{\text{cullis}} = \sum_h |F_D(h) - |F_N(h)| + |F_H(h)|| / \sum_h |F_D(h) - F_N(h)|$, where $F_H(h)$, $F_D(h)$ and $F_N(h)$ are the heavy atom, derivative and native structure factor amplitudes of reflection *h*.

^d Phasing power = $\sum_h |F_H(h)| / \sum_h |F_D(h) - |F_N(h)| + F_H(h)||$.

^e $\sum_h ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_h |F_{\text{obs}}|$, where $|F_{\text{obs}}|$ and $|F_{\text{calc}}|$ are the observed and calculated structure factor amplitudes for the reflection *h*, applied to the working (*R*_{cryst}) and test (*R*_{free}) sets, respectively.

^f Non-hydrogen atoms, only.

^g rmsd: root-mean-square deviation from mean.

A binary complex of the Endo-F1 glycosidase treated sNEP at 20 mg/ml and the inhibitor phosphoramidon at a concentration of 1 mM was formed. Crystals of the inhibited protein complex were obtained by vapor diffusion with 25% PEG 3350, 200 mM ammonium sulfate 100 mM bis-Tris at pH 7.5 as the precipitating agent. Crystals belong to the trigonal space group *P*₃21 with one molecule in the asymmetric unit. All crystals were flash frozen in a cryoprotectant solution corresponding to the reservoir condition containing 30% (v/v) glycerol and measured at 120 K. All data sets, except Native-2, were measured with CuK α radiation provided from a NONIUS FR591 rotating anode generator equipped with an OSMIC mirror system, recorded on a MAR-Research image plate area detector and evaluated with the XDS software (Kabsch, 1988). The Native-2 data set, used for refinement, was collected at the Swiss-Norwegian beam line (SNBL) at the European Synchrotron Radiation Facility (ESRF, Grenoble) on a MAR image plate at 0.86633 Å. Data were processed and scaled with DENZO and SCALEPACK (Otwinowski, 1993). All data sets were further analyzed with the CCP4 program suite (CCP4, 1994). A EuCl₃ derivative was solved from the isomorphous amplitude differences relative to Native-1 using the program SHELXS-97 (Sheldrick *et al.*, 1993). Three sites were identified and initial phases obtained from the isomorphous and anomalous differences of this derivative were used to solve a mercury and a uranyl derivative by difference Fourier methods. All heavy atom refinement and phasing was performed with the program SHARP (de La Fortelle & Bricogne, 1997). The initial SHARP electron density was modified and further improved with the program SOLOMON by applying iterative rounds of solvent-flipping and a final cycle of solvent-flattening using a solvent content of 56% (Abrahams & Leslie, 1996). This led to a traceable electron density for about 90% of the structure at 2.5 Å resolution. Model building was performed with the in-house graphics program MOLOC, stereochemically restrained positional and temperature-factor refinement was done with CNS 0.5 against the amplitude based maximum likelihood target “MLF1”, using parameters for ideal stereochemistry as described by Engh & Huber and a bulk solvent correction (Brünger *et al.*, 1998; Engh & Huber, 1991; Gerber, 1992; Jiang & Brünger, 1994; Kostrewa, 1997). Iterative rounds of model building and refinement led to a model (lacking the N-terminal two residues D52 and D53) with excellent stereochemistry as examined by PROCHECK and converged to an *R*-factor of 0.194 using all data in the resolution range between 20.0 and 2.1 Å (42,045 reflections) with an *R*_{free} of 0.242 (2210 reflections) (Brünger, 1992; Laskowski *et al.*, 1993). All residues fall within the allowed regions of the Ramachandran Plot, with the exception of L199, which has a clearly defined backbone conformation. All Figures were generated with the programs MOLSCRIPT, RASTER3D, or GRASP (Kraulis, 1991; Merrit & Bacon, 1997; Nicholls & Honig, 1992).

has been implicated in the stabilization of the transition state (Dion *et al.*, 1995). Furthermore, this residue interacts with D709 and stabilizes a complex hydrogen bonding network, similar to

that observed in TLN. This network also involves the residues E584, R717, as well as the tri-peptide ⁵⁴¹VNA⁵⁴³. In the crystal structure E584 is hydrogen bonded to the second oxygen

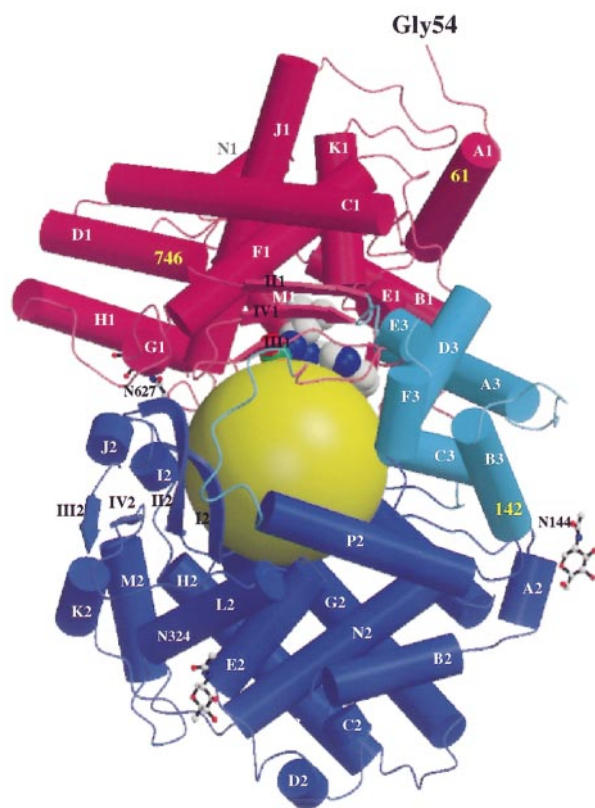


Figure 4. Ribbon plot of sNEP depicting the volume of the active site cavity.

atom of the tetrahedral N-phosphoryl moiety of the inhibitor (O2) with an inter-atomic distance of 2.73 Å and is in position to be involved in catalysis by polarizing the attacking water molecule (Devault *et al.*, 1988a). The side-chain of R717 participates in a salt bridge with D650 and plays a role in substrate hydrolysis and inhibitor binding (Marie-Claire *et al.*, 1997). It interacts with the inhibitor by forming a hydrogen bond with the carbonyl oxygen atom of the L-leucyl residue in the P₁' position. Additionally, the carbonyl oxygen atom of A543 is hydrogen bonded with the peptide amide group of the P₁' residue of phosphoramidon. The side-chain of N542 forms two hydrogen bonds with the NH and CO groups of the P₂' residue of the inhibitor with hydrogen-bonding distances of 3.27 Å and 3.20 Å, respectively, and thus plays an analogous role to that of N112 in TLN (Dion *et al.*, 1995). The indole NH of the L-tryptophan residue is hydrogen bonded to the main-chain carbonyl group of V541 (2.83 Å).

The rhamnose moiety of the P₁ residue of the inhibitor is mainly exposed to solvent, leaving 80% of the surface of phosphoramidon in direct

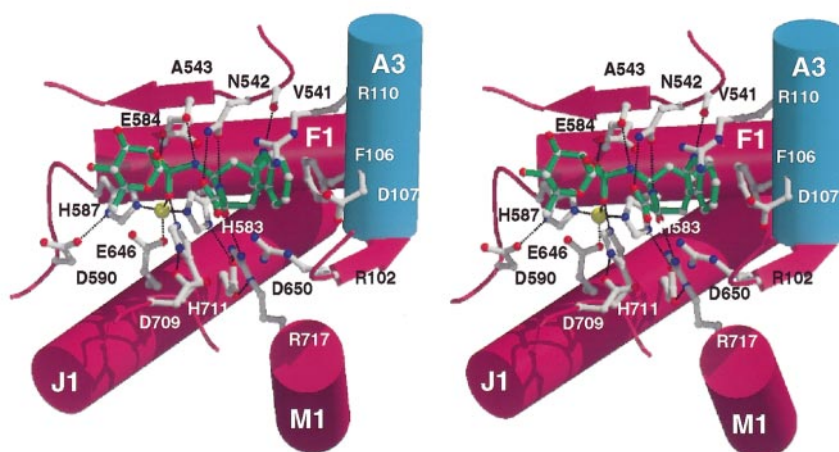
contact with the enzyme (Figure 5). This reflects the minor stabilizing role of the S₁ subsite expected by the slight increase in affinity of inhibitors with a P₁ side-chain (Gaucher *et al.*, 1999; Ksander *et al.*, 1997a,b). The absence of a stringent specificity for the S₁ subsite is in contrast to that observed for the binding site of the P₁' residue. The S₁' subsite of sNEP forms a large hydrophobic pocket that interacts preferentially with aromatic or large hydrophobic moieties (Tiraboschi *et al.*, 1999). It is formed by the side-chains of F106, I558, F563, M579, V580, V692 and W693. This subsite has a volume of approximately 220 Å³ and is only partially filled by the L-leucyl side-chain of the inhibitor. The indole moiety of the P₂' residue of the inhibitor is in van der Waals contact to the side-chain of F106. The S₂' subsite is large and extends to the solvent across the linker helix A3 towards the side-chains of R102, D107 and R110. This confirms the observation that the S₂' subsite of sNEP has reduced specificity and can accommodate bulky side-chains. None of these charged residues is involved in inhibitor binding. Moreover, the guanidinium group of both arginine residues is disordered in the otherwise well-defined active site. This is consistent with the role of R102 in substrate binding believed to be dependent on the geometry and the nature of the P₂' residue (Kim *et al.*, 1992). Interestingly, no residue of domain 2 is involved in inhibitor binding or subsite formation.

This is, to our knowledge, the first three-dimensional structure of a member of this pharmacologically important family of proteins. Among the wide range of biologically active peptide substrates for NEP, the enkephalins and the atrial natriuretic peptide have assumed greatest significance. They reflect the important role of this membrane peptidase in the modulation of nociceptive and pressor responses which has stimulated considerable interest in inhibitors of this enzyme to treat a variety of disease states including acute pain and hypertension. In the background of this rich biological context further understanding of NEP is poised to benefit from this structural study. The information provided by the three-dimensional structure presents an attractive challenge to increase the potency and selectivity of known inhibitor classes through docking studies. Moreover, this structure provides a tool for further characterization of other members of the neprilysin subfamily, such as ECE.

Protein Data Bank accession numbers

The NEP coordinates have been deposited in the RCSB Protein Data Bank (PDB code 1DMT).

a



b

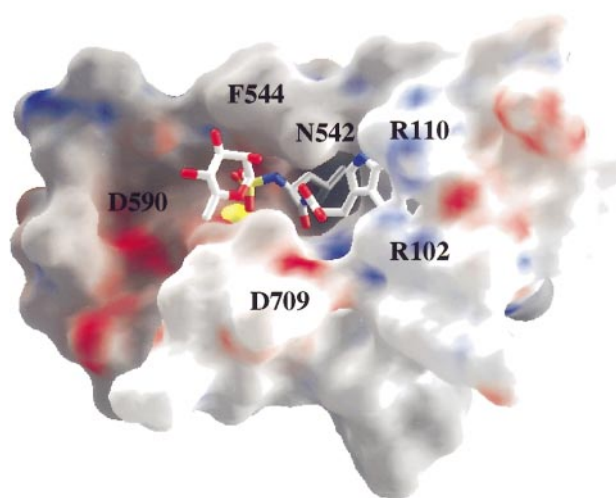


Figure 5. Inhibitor binding to the active site of human sNEP. (a) Stereo view of the active site inhibited by phosphoramidon, green. Hydrogen bonds and ionic interactions are indicated by broken lines, α -helices are shown as cylinders, β -strands are represented by arrows: catalytic domain, violet; the smaller domain 2, blue; the three interdomain linker fragments, cyan. The zinc ion is shown in yellow. (b) Molecular surface of the phosphoramidon recognition site colored by electrostatic potential: positive, blue; negative, red.

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Exhibit 31

Redacted in its Entirety

**CERTAIN DRUG PRODUCTS
CONTAINING C-TYPE NATRIURETIC
PEPTIDE VARIANTS, AND
COMPONENTS THEREOF**

Inv. No. 337-TA-1447

CERTIFICATE OF SERVICE

I, hereby certify that on this day, February 5, 2026 a true and correct copy of the foregoing **PUBLIC VERSION OF RESPONDENTS' REPLY CLAIM CONSTRUCTION BRIEF** was filed and served upon the following as indicated:

The Honorable Lisa R. Barton Secretary to the Commission U.S. International Trade Commission 500 E Street, S.W., Room 112 Washington, DC 20436	☒ Via EDIS ☐ Via Hand Delivery ☐ Via First Class Mail ☐ Via Electronic Mail ☐ Via Overnight Courier
The Honorable Clark S. Cheney Chief Administrative Law Judge U.S. International Trade Commission 500 E Street, S.W., Room 112 Washington, DC 20436 Cheney1447@usitc.gov	☐ Via Hand Delivery ☐ Via First Class Mail ☒ Via Electronic Mail ☒ Via Box
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/s/ Russell W. Warnick
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Litigation Paralegal