Eur. J. Biochem. 219, 73–81 (1994)
© FEBS 1994

Genuine and apparent cross-reaction of polyclonal antibodies to proteins and peptides

Lukas LEDER, Hans WENDT, Christian SCHWAB, Ilian JELESAROV, Susanne BORNHAUSER, Friederike ACKERMANN and Hans Rudolf BOSSHARD

Biochemisches Institut der Universität, Zürich, Switzerland

(Received June 17/September 22, 1993) – EJB 93 0905/3

Antiserum to a native protein may cross-react with the corresponding denatured protein or with peptides. The cross-reaction is either a genuine property of the antibodies or caused by antibodies produced against some unfolded protein contaminating the native protein used for immunization. Appropriate conformation-sensitive immunoassays must be employed to distinguish a genuine from an apparent cross-reaction.

In the present study, we have analyzed critically the cross-reaction of rabbit antisera against proteins and peptides. We have distinguished between genuine and apparent cross-reaction with the help of the protein A antibody-capture ELISA, a new conformation-sensitive ELISA format. Three systems were analyzed: cross-reaction of antisera to native yeast and horse cytochrome *c* with unfolded apo-cytochrome *c*; cross-reaction of antisera to a coiled-coil leucine-zipper peptide with a homologous random-coil peptide obtained by introducing two proline residues into the leucine-zipper sequence; cross-reaction of antisera to two peptides that correspond to the N-terminal and an internal sequence of ferredoxin:$NADP^+$ reductase (FNR), with the native enzyme. The reaction of the anti-(cytochrome *c*) sera was clearly due to antibodies produced against unfolded protein, it was an apparent and not a genuine cross-reaction. Furthermore, the apparently cross-reactive antibodies to horse cytochrome *c* did not discriminate against sequence-related proteins from dog, beef, rabbit and pigeon. In contrast, antibodies to the leucine-zipper peptide did cross-react in a genuine way with the homologous random-coil peptide, that is, the cross-reactive antibodies do not seem to have been produced against the unfolded form of the leucine-zipper peptide. Of the two anti-peptide sera the one against the unstructured and highly accessible N-terminal segment reacted strongly with the native protein. The second serum against a solvent-accessible turn-like sequence of FNR showed apparent cross-reactivity: antibodies recognizing the native protein were directed against a minor conformational isoform of the free peptide and did not react with the principal form(s) of the free peptide.

The generation of cross-reactive antibodies depends on the conformational stability and intergrity of the immunogen and on the molecular form of its application, i.e., free, polymerized or carrier-bound. The results clarify the different nature of cross-reactivity of antisera to proteins and peptides. This knowledge is crucial if antisera are to be used as conformation-specific probes.

The cross-reaction of an anti-protein serum with the denatured protein can have two reasons. First, the cross-reaction may be a genuine property of antibodies that are directed primarily against the native structure. Second, the cross-reaction can be caused by unfolded forms of the protein present in the material used for immunization. For example, the reaction of anti-lysozyme sera with denatured and carboxymethylated lysozyme was ascribed many years ago to contamination of the immunogen with denatured protein (Scibienski, 1973). The cross-reactive antibodies were directed against the contaminant(s) rather than against the parent immunogen. We call this type of cross-reaction apparent.

There have been few studies, most of them published years ago, in which the cross-reaction of antisera induced by native and denatured proteins was analyzed. Much of the early work was on lysozyme, myoglobin, ribonuclease and chemical derivatives of these proteins (review by Brawn and Dandliker, 1977). The concepts of cross-reactivity of antibodies to proteins and peptides were discussed by Berzofsky and Schechter (1981).

Genuine cross-reaction of anti-protein antibodies with the denatured protein or with peptides is rare, as already noted by Landsteiner (1945). More recently, Jemmerson showed that the vast majority of several hundred monoclonal antibodies to rat cytochrome *c* does not recognize CNBr-generated fragments of the protein, the few cross-reacting antibodies being most probably directed against the unfolded protein (Jemmerson, 1987). The occurrence of apparent cross-reac-

Correspondence to H. R. Bosshard, Biochemisches Institut der Universität, Winterthurerstr. 190, CH-8057 Zürich, Switzerland
Fax: +41 1 363 7947.

Abbreviations. FNR, ferredoxin:$NADP^+$ oxidoreductase; PACE, protein A antibody-capture ELISA.

Enzyme. Ferredoxin:$NADP^+$ oxidoreductase (EC 1.18.1.2).

Note. L. Leder and H. Wendt contributed equally to this work.

Petitioner Merck, Ex. 1163, p. 73
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00006

tivity by anti-protein antibodies was demonstrated unequivocally by the isolation of monoclonal antibodies that react exclusively with the denatured protein even though the animals were immunized with the native protein (e.g., Friguet et al., 1984).

The first use of peptides to produce protein-reactive antibodies was a landmark in the history of immunochemistry (Anderer, 1963). Nowadays, anti-peptide antibodies have found vast application in biology and medicine, for example as probes in gene-cloning procedures or as synthetic vaccines (Arnon and Van Regenmortel, 1992). A peptide must mimic structural features of the native protein fold to induce protein-reactive antibodies. There have been many attempts to screen polypeptide sequences for segments that may be antigenic and produce protein-reactive antibodies if presented to the immune system in the form of synthetic peptides (Pellequer et al., 1991). The success of prediction methods has been equivocal. Moreover, reports about anti-peptide antibodies reactive with the native protein were sometimes mistaken because the protein used in the test was partially unfolded and the cross-reaction took place with the unfolded protein (Spangler, 1991). Some authors have challenged the view that antibodies to peptides may recognize the corresponding native proteins at all (Laver et al., 1990). However, the successful production of virus-neutralizing antibodies by immunization with peptides strongly suggests that genuine cross-reaction with native proteins is possible (Arnon, 1987; Arnon and Van Regenmortel, 1992).

In the present study, we have analyzed critically the nature of the cross-reaction of antisera to proteins and peptides. We have been able to distinguish between genuine and apparent cross-reaction by a new competition ELISA in which antigen and antibody equilibrate in solution without the danger of denaturation of the protein antigen (Ngai et al., 1993).

EXPERIMENTAL PROCEDURES

Materials

Yeast (*Saccharomyces cerevisiae*) iso-1 cytochrome *c*, horse cytochrome *c* and the corresponding apo-cytochromes *c* were prepared as before (Saad et al., 1988; Savoca et al., 1991). Contamination of apo-cytochrome *c* with intact cytochrome *c* was below 0.1%, which was the limit detectable by absorbance spectroscopy and heme staining in polyacrylamide gel electrophoresis. Yeast cytochrome *c* (1.33 mM) was polymerized with glutaraldehyde (8.5 mM) in 0.1 M sodium phosphate, pH 7.0, for 10 min. The reaction was stopped by addition of excess lysine hydrochloride, and the material was dialyzed against phosphate buffered saline. Glutaraldehyde-polymerized cytochrome *c* was soluble but did not penetrate into a 10% polyacrylamide gel. Horse cytochrome *c* and apo-cytochrome *c* were coupled to succinylated keyhole limpet hemocyanin (Pierce) for immunization (Kuo and Davies, 1983). Ferredoxin:$NADP^+$ oxidoreductase (FNR) was prepared from spinach chloroplasts as described by Shin and Oshino (1978).

Biotinylation of cytochrome *c* and FNR

This was performed as before (Ngai et al., 1993). The degree of biotinylation was 0.1−0.2 mol/mol for yeast cytochrome *c* and 0.9 mol/mol for horse cytochrome *c* and FNR.

Peptide synthesis

The following peptides were synthesized: EWEALEKK-LAALEAAKQALEKKLEALEHG, LZ; biotinyl-GGG-E-WEALEKKLAALEAKLQALEKKLEALEHG, biotinyl-LZ;biotinyl-GGG-C-GGG-EWEALEKKLAALEAKLQAL-EKKLEALEHG, biotinyl-LZ-disulfide; EWEALEPKLAA-LEPKLQALEKKLEALEHG, LZ7P14P; botinyl-GGG-E-WEALEPKLAALEPKLQALEKKLEALEHG, biotinyl-LZ7P14P; Ac-YQIASDVEAPPPAPAKVEKHS, FNR(1−20); biotinyl-GGG-Y-GGG-QIASDVEAPPPAPAKVEKHS, biotinyl-FNR(1−20); Ac-NKFKPKTPYV-NH_2, FNR(30−39); biotinyl-GGG-NKFKPKTPYV-NH_2, biotinyl-FNR(30−39); Ac-TGQAPGFTYTDA-NH_2, HCC(40−51); Ac-KGIT-WKEETLME-NH_2, HCC(55−66); Ac-IKKKTEREDLIAY-NH_2, HCC(85−97). Dashes are added for clarity to separate the main sequence of a peptide from triglycine spacer, additional cysteine or tyrosine residue, and N-terminal and C-terminal protection groups. Residues in boldface are in positions a and d of heptad repeats of leucine zippers. Positions in which peptides LZ and LZ7P14P differ are underlined, positions in which horse and rabbit cytochrome *c* differ are double underlined. Tyr was added as a spectroscopic marker to FNR(1−20) and biotinyl-FNR(1−20). Peptides LZ, LZ7P14P, FNR(1−20) and their N^{α}-biotinyl derivatives were synthesized on an Applied Biosystems synthesizer Model 430A. The N^{α}-9-flurenylmethyloxycarbonyl protection strategy was used. Synthesis was performed on a *p*-alkoxybenzyl polystyrene resin, yielding a free C-terminal residue. Peptide biotinyl-LZ-disulfide was obtained by oxidation of cysteine with $K_3Fe(CN)_6$. Peptides FNR(30−39), HCC(40−51), HCC(55−66), and HCC(85−97) were synthesized on a RaMPS synthesizer from DuPont, as described (Savoca et al., 1991). The peptides produced on the RaMPS synthesizer were N^{α}-acetyl protected and contained a C-terminal amide group. Synthetic peptides were purified by reverse-phase HPLC on a C_8-column with a solvent system of 0.1% trifluoroacetic acid and an increasing gradient of acetonitrile (Saad et al., 1988). HPLC-purified peptides were analyzed by ion-spray mass spectrometry. Impurities amounted to less than 2%.

Preparation of antisera

Female New Zealand white rabbits were immunized initially with 100 µg protein or peptide emulsified in complete Freund's adjuvant. Animals were given booster injections at intervals of 2−4 weeks with 100 µg immunogen in incomplete adjuvant. Serum was collected 1 week after each of a total of 3−5 boosts. All experiments were performed with antibodies from the third or later bleeding. In the case of sera N4, N5 and A1 (Fig. 1), whole serum as well as protein-A-purified IgG fractions were used. The results with whole serum and with purified IgG were qualitatively the same. The data presented in Fig. 1 are for whole sera. The IgG fraction of sera against peptides FNR(1−20) and FNR(30−39) were used in the experiments shown in Fig. 5. IgG was isolated by chromatography on protein-A−Sepharose (Pierce).

One rabbit each was immunized with peptides LZ, LZ7P14P, biotinyl-LZ-disulfide, biotinyl-FNR(1−20) and biotinyl-FNR(30−39). Avidin complexes of the biotinylated peptides were used for immunization (Scott et al., 1984). Two rabbits were immunized with native cytochrome *c* from the horse (sera NH1 and NH2) and two rabbits with the corresponding apo-cytochrome *c* (sera AH1 and AH2). Horse

Petitioner Merck, Ex. 1163, p. 74
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00006

cytochrome *c* coupled to keyhole limpet hemocyanin was used for immunization. The antisera against yeast iso-1 cytochrome *c* were those used in our previous studies: sera N1, N3 and N4 against glutaraldehyde-polymerized native cytochrome *c*, sera N5 and N6 against monomeric native cytochrome *c* (Schwab et al., 1993), and sera A1, A3 and A4 against monomeric apo-cytochrome *c* (Schwab and Bosshard, 1992).

Protein A antibody-capture ELISA (PACE)

Details of the assay have been published (Ngai et al., 1993). Briefly, microtiter plates (Nunclon MicroWell no. 1-67008A) were coated at 4°C overnight with 50 µl/well protein A (Pharmacia) dissolved (20 µg/ml) in coating buffer (15 mM Na_2CO_3, 34.8 mM $NaHCO_3$, 0.02% NaN_3, pH 9.6). Plates were washed with NaCl/P_i/T (1.46 mM KH_2PO_4, 6.46 mM Na_2HPO_4, 0.14 M NaCl, 0.27 mM KCl, 0.02% NaN_3 0.5% Tween-20, pH 7.2) and blocked with blocking buffer (2% fat-free dried milk in NaCl/P_i/T) for 1 h at 37°C. After washing with NaCl/P_i/T, plates were ready to capture the previously formed antigen-antibody complex. This complex was prepared by incubation of biotinylated tracer antigen, serially diluted competitor antigen, and appropriately diluted antiserum, for 1 h at room temperature. Concentrations of tracer antigens were 0.1−0.3 µM. The antibody dilution was chosen such that in the absence of competitor, 60−80% of maximum absorbance was reached. Solutions were transferred to the protein-A-coated microtiter plate and incubated for 1 h at 37°C. Plates were washed with NaCl/P_i/T and developed with streptavidin-alkaline phosphatase conjugate (Boehringer) and *p*-nitrophenylphosphate as substrate. Colour development was followed at 405 nm with an ELISA reader (Bio Rad). Control assays performed without serum, with pre-immune serum, or without second antibody were negative. Sera produced with avidin-bound peptides were tested for reaction with streptavidin, which was used as the detection reagent in the PACE. No reaction was seen with streptavidin bound to the microtiter plate, except for a weak reaction in the case of the serum to FNR(30−39). This serum was affinity-purified on streptavidin before use in the experiments described.

Other methods

RIA with ^{125}I-labelled tracer antigen and solid-phase ELISA were performed as before (Schwab and Bosshard, 1992). Concentrations of cytochromes were determined photometrically using the following absorption coefficients: $\varepsilon_{550(red\text{-}ox)}$ 19 mM^{-1} cm^{-1} (native cytochrome *c*); ε_{280} 10.9 mM^{-1} cm^{-1} (horse apo-cytochrome *c*); ε_{280} 11.6 mM^{-1} cm^{-1} (yeast apo-cytochrome *c*). Concentrations of peptides were calculated from amino acid analyses. CD spectra were measured on a Jasco J-500 C spectropolarimeter and secondary-structure content was calculated as in Chen et al. (1972).

RESULTS

Criteria for genuine and apparent cross-reaction

An antiserum against a protein is highly heterogeneous. Every individual antibody not only binds to the protein that is used as the immunogen, but may also bind to other protein molecules. This heterogeneity is thought to result in a higher specificity of the whole antiserum: while some single antibody may be only moderately specific, the whole serum is highly specific for the one antigen common to all the antibodies in the serum (Talmadge, 1959). This common antigen is referred to as the cognate antigen. Consequently, any subpopulation of antibodies able to genuinely cross-react with a conformational isoform of the immunogen, referred to as the non-cognate antigen, is still primarily directed against the cognate antigen. It also follows that genuine cross-reactivity is not a property of a whole serum but of some of its component antibodies (Berzofsky and Schechter, 1981).

Based on these considerations, we use as the criterion for genuine cross-reaction a higher avidity for the cognate antigen than for the non-cognate antigen. The opposite applies for apparent cross-reactivity: higher avidity for the non-cognate antigen than for the cognate antigen. The reason is, of course, that the apparent cross-reaction is due to antibodies induced by and directed against altered conformational isoforms.

Competitive immunoassay to test type of cross-reaction

Specificity for different conformational isoforms must be tested in a solution-phase immunoassay because antigen and antibody may denature in a solid-phase assay through adsorption to the solid surface (Van Regenmortel, 1989; Schwab and Bosshard, 1992). We have developed the conformation-sensitive ELISA procedure PACE (Ngai et al., 1993). In this assay, a biotinylated antigen and antibodies are first allowed to equilibrate in solution. Thereafter, the antigen-antibody complexes (and free antibody) are captured to a microtiter plate that has been coated with protein A. Protein-A-captured antigen-antibody complexes are then detected by streptavidin-alkaline phosphatase conjugate and *p*-nitrophenylphosphate as substrate. The competition PACE is akin to a classical competition RIA except that radioiodinated tracer antigen is exchanged for biotinylated tracer antigen. In the competition PACE, the antibodies are incubated in solution with a constant amount of biotinylated antigen (called tracer antigen) and increasing amounts of non-biotinylated competitor. After reaching equilibrium, the antigen-antibody complexes are captured by the protein-A-coated microtiter plate and detected with streptavidin-enzyme conjugate.

The competition PACE was used to test for genuine and apparent cross-reactivity. By switching from cognate to non-cognate tracer antigen one selects for different populations of cross-reacting antibodies. We could distinguish between genuine and apparent cross-reaction in the following way. The cognate antigen was a stronger competitor than the non-cognate antigen if the cross-reaction was genuine, independent of whether the cognate or non-cognate antigen was used as the biotinylated tracer. In the case of apparent cross-reaction, the non-cognate antigen was a stronger competitor and the cognate antigen a weaker competitor if the tracer antigen was non-cognate. In the reverse test with the cognate tracer antigen, the cognate antigen was a stronger and the non-cognate antigen a weaker competitor. The principle is illustrated by an example; antibodies to a native protein cross-react with the denatured protein in a genuine way if the native protein is a better competitor, independent of the tracer antigen. The antibodies cross-react in an apparent manner if the denatured protein is a better competitor in the test with the denatured tracer antigen but not in the test with the native tracer antigen.

Petitioner Merck, Ex. 1163, p. 75
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00006

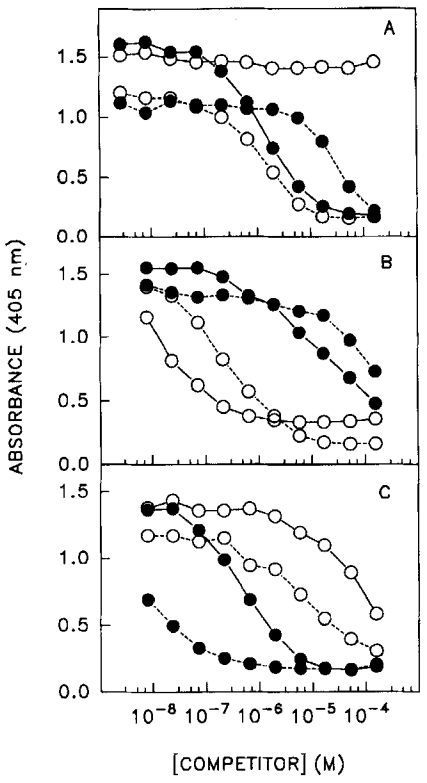


Fig. 1. Cross-reaction of antibodies to iso-1 cytochrome *c* and apo-cytochrome *c* from yeast. In A and B of this figure as well as in Figs 2A, 4A, B and 5, line types and symbols have the following general meaning. Solid lines, tracer is biotinylated cognate antigen. Dashed lines, tracer is biotinylated non-cognate antigen. Filled symbols, competitor is cognate antigen. Open symbols, competitor is non-cognate antigen. (A) Antiserum N4 (1:2000) against glutaraldehyde-polymerized native yeast cytochrome *c* assayed with biotinyl-cytochrome *c* (cognate tracer antigen, solid lines) or biotinyl-apo-cytochrome *c* (non-cognate tracer antigen, dashed lines) in the presence of increasing amounts of cytochrome *c* (cognate competitor, ●) or apo-cytochrome *c* (non-cognate competitor, ○). Cross-reaction is apparent as revealed by the curve-shift to lower competitor concentration when the serum is assayed with non-cognate tracer and non-cognate competitor (dashed line with open symbols). (B) Antiserum N5 (1:400) against monomeric native yeast cytochrome *c* assayed with biotinyl-cytochrome *c* (cognate tracer antigen, solid lines) or biotinyl-apo-cytochrome *c* (non-cognate tracer antigen, dashed lines) in the presence of increasing amounts of cytochrome *c* (cognate competitor, ●) or apo-cytochrome *c* (non-cognate competitor, ○). This serum contains a major fraction of antibodies reacting preferentially with non-native form(s) of cytochrome *c*. (C) Antiserum A1 (1:400) against monomeric yeast apo-cytochrome *c* assayed with biotinyl-apo-cytochrome *c* (cognate tracer antigen, solid lines) or biotinyl-cytochrome *c* (non-cognate tracer antigen, dashed lines) in the presence of increasing amounts of apo-cytochrome *c* (cognate competitor, ●) or cytochrome *c* (non-cognate competitor, ○). Cross-reaction is genuine. Tracer concentration was 0.1 μM in all experiments.

Antibodies to native and apo-cytochrome *c* from yeast

Yeast iso-1 cytochrome *c* is a small (M_r 12588) globular protein with a covalently linked heme prosthetic group. Removal of the heme produces random coil apo-cytochrome *c* (Damaschun et al., 1991). Rabbits were immunized with monomeric native or apo-cytochrome *c*, or with glutaraldehyde-polymerized native cytochrome *c*. Polymerization enhances immunogenicity (Reichlin et al., 1970). Fig. 1 shows competitive immunoassays obtained with antisera N4, N5 and A1. Antiserum N4 raised against glutaraldehyde-polymerized native yeast cytochrome *c* was highly specific for the cognate native antigen. Non-cognate antigen did not compete for binding to the cognate tracer antigen, which was biotinylated native cytochrome *c* (Fig. 1A). However, when the tracer was changed to biotinylated apo-cytochrome *c*, the non-cognate antigen apo-cytochrome *c* proved to be a better competitor than native cytochrome *c*. This clearly indicated that the cross-reaction was not genuine. The same results were observed with antisera N1 and N3 to glutaraldehyde-polymerized native cytochrome *c* (data not shown).

An interesting observation was made with sera N5 and N6 induced by monomeric native yeast cytochrome *c*. These sera, the titers of which were low, as was expected for small monomeric cytochrome *c* (Reichlin et al., 1970), reacted more strongly with the non-cognate antigen apo-cytochrome *c* even when the cognate antigen biotinyl-cytochrome *c* was used as the tracer (results for serum N5 shown in Fig. 1B). We have to assume that a dominant fraction of the antibodies to the non-polymerized monomeric yeast cytochrome *c* was actually directed against a non-native form of cytochrome *c*. We can rule out that biotinylated native cytochrome *c* used as the tracer was itself denatured and thereby caused this unexpected result. The degree of biotinylation was only 0.1–0.2 mol/mol, hence very few molecules had more than one modified lysine. This low degree of modification does not change the native conformation of cytochrome *c* (Michel et al., 1989). Moreover, had biotinyl-cytochrome *c* been denatured, preference for apo-cytochrome *c* would have been seen with antibodies to the polymerized yeast cytochrome *c* shown in Fig. 1A. The preference of serum N5 for the apo-form was confirmed by a conventional RIA. Titers were 1:50000 for ^{125}I-labelled apo-cytochrome *c* and only 1:3000 for ^{125}I-labelled native cytochrome *c*.

Fig. 1C depicts the results for serum A1 to yeast apo-cytochrome *c*. Here, the cognate antigen apo-cytochrome *c* was a better competitor independent of the biotinylated tracer antigen employed, hence the cross-reaction was genuine. The cross-reaction of antibodies to a denatured protein with the native protein must be genuine because the denatured immunogen cannot fold back to the native protein and induce apparently cross-reacting antibodies.

Antibodies to native and apo-cytochrome *c* from the horse

Native and apo-cytochrome *c* were coupled to keyhole limpet hemocyanin as carrier immunogen. Linkage was through ε-amino groups of cytochrome *c*. Antisera NH1 and NH2 showed almost absolute specificity for native horse cytochrome *c* when tested with the cognate tracer biotinyl-cytochrome *c* (Fig. 2A). This high conformational specificity is in agreement with earlier reports (Jemmerson and Margoliash, 1979; Jemmerson, 1987). However, by changing to the non-cognate tracer antigen (biotinylated apo-cytochrome *c*), we were able to demonstrate the presence of cross-reacting antibodies in sera NH1 and NH2 against native horse cytochrome *c*. As expected, the cross-reaction was only apparent; apo-cytochrome *c* was a very much stronger competitor than native cytochrome *c* (Fig. 2A).

Genuine cross-reaction with native horse cytochrome *c* was displayed by antisera AH1 and AH2 against horse apo-cytochrome *c* (data not shown).

Petitioner Merck, Ex. 1163, p. 76
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00006

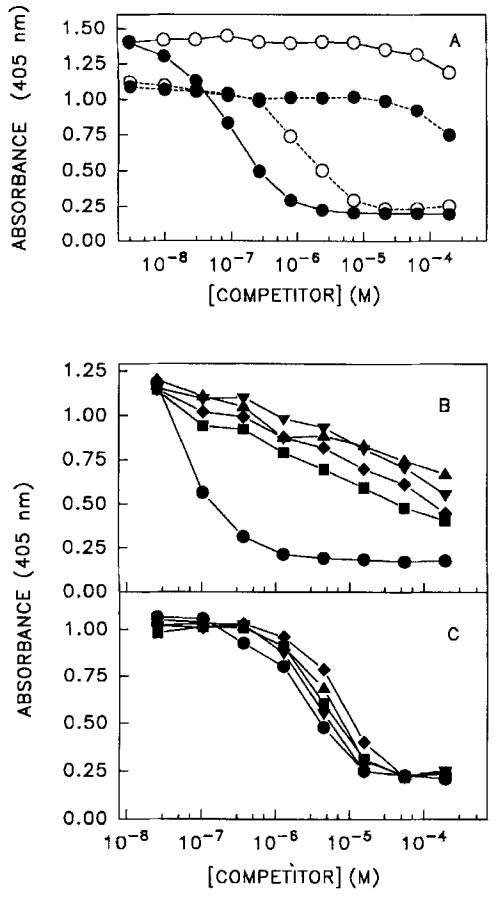


Fig. 2. Cross-reaction of antibodies to native cytochrome *c* from the horse. (A) Antiserum NH1 (1:80) against horse cytochrome *c* was assayed with biotinyl-cytochrome *c* (cognate tracer antigen, solid lines) or biotinyl-apo-cytochrome *c* (non-cognate tracer antigen, dashed lines) in the presence of increasing amounts of cytochrome *c* (cognate competitor, ●) or apo-cytochrome *c* (non-cognate competitor, ○). The cross-reaction with horse apo-cytochrome *c* is of the apparent type. (B) Species specificity of antiserum NH1 (1:80) against horse cytochrome *c* assayed with biotinylated native cytochrome *c* as tracer antigen. Competitor antigens were: cytochrome *c* from horse (●), beef (■), dog (◆), rabbit (▲), and pigeon (▼). (C) Same as (B) but assayed with biotinylated horse apo-cytochrome *c* as tracer antigen. Tracer concentration was 0.1 μM in all experiments.

Species specificity of antibodies against horse cytochrome *c*

Anti-(cytochrome *c*) antibodies discriminate strongly against other sequence-related vertebrate cytochromes *c* (Urbanski and Margoliash, 1977; Jemmerson and Margoliash, 1979; Benjamin et al., 1984). Fig. 2B exemplifies the species-specificity of serum NH1 in tests with cytochrome *c* from beef (3 sequence differences), rabbit (6 sequence differences), dog (5 sequence differences) and pigeon (11 sequence differences). However, this specificity was almost completely lost in the test with the non-cognate tracer, biotinylated apo-cytochrome *c* (Fig. 2C). The sequence differences were recognized only in the context of the native cytochromes. When the specificity of serum AH1 against horse apo-cytochrome *c* was tested with several peptide fragments, peptide 1–38 was a better competitor than any of the peptides containing sequence differences between rabbit and horse cytochrome *c* (Table 1). The sequence differences between horse and rabbit cytochrome *c* are at position 44 (Pro/Val), 47 (Thr/Ser), 60 (Lys/Gly), 62 (Glu/Asp), 89 (Thr/Asp) and 92 (Glu/Ala). A mixture of the synthetic peptides HCC(40–51), HCC(55–66), and HCC(85–97), each containing two of the six sequence differences, did not compete with binding of serum AH1 to biotinylated apo-cytochrome *c* (Table 1). This shows again that antibodies to the unfolded cytochrome cannot distinguish species differences.

Table 1. Competition immunoassay with serum AH1 against horse apo-cytochrome *c*. Tracer antigen was 0.1 μM biotinylated horse apo-cytochrome *c*, serum dilution was 1:80.

Competitor antigen	Concentration at 50% inhibition
	mol/l
Apo-cytochrome *c*	5×10^{-8}
Peptide 1–65	5×10^{-8}
Peptide 1–38	1×10^{-6}
Peptide 39–65	approx. 10^{-5}
Peptide 66–80	2×10^{-4}
Peptide 81–104	1×10^{-5}
HCC(40–51) + HCC(55–66) + HCC(85–97)	$\gg10^{-4}$[a]

[a] Equimolar mixture.

Antibodies to a coiled-coil leucine zipper

The sequence of peptide LZ was designed by O'Neil and DeGrado (1990) and was used by these authors to assess the helix-forming tendencies of amino acids through permutation of the residue in position 14. Peptide LZ consists of four heptad repeats after the N-terminal Glu (see Experimental Procedures for sequences of peptides). Positions a and d of each heptad is Leu, except for Trp in the first heptad. The residues in a and d contribute to the formation of the coiled-coil structure. Peptide LZ forms a very stable coiled-coil. It is not clear whether in solution the coiled-coil is dimeric (O'Neil and DeGrado, 1990) or trimeric (Lovejoy et al., 1993).

The CD spectrum of peptide LZ indicates greater than 95% α-helix content (Fig. 3A). Introduction of two prolines in positions 7 and 14 of peptide LZ7P14P collapses the coiled-coil (Fig. 3A). Urea denaturation of peptide LZ occurred at a midpoint concentration of 5.5 M (Fig. 3B). Peptide biotinyl-LZ was even more stable with a midpoint concentration of 7.5 M urea (Fig. 3B). The higher stability may be due to protection of the positive N-terminal charge at the helix end in biotinyl-LZ (Scholtz and Baldwin, 1992).

Rabbits were immunized with the free peptides LZ and LZ7P14P or with the covalent dimer biotinyl-LZ-disulfide. In the latter peptide, the disulfide bond is separated from the coiled-coil by a triglycine spacer. The disulfide bond further stabilizes the coiled-coil structure (Hodges et al., 1988). The far ultraviolet CD spectrum of peptide biotinyl-LZ-disulfide indicated again greater than 95% helical content (data not shown). Introduction of biotinyl groups, separated from the disulfide bond by another triglycine spacer, allowed to couple the peptide to avidin. Biotinyl-LZ-disulfide bound to avidin was used for immunization. Avidin acted as a carrier, leading to much higher antibody titers than in the case of the serum against free peptides LZ and LZ7P14P (see antibody dilutions in legend to Fig. 4).

Petitioner Merck, Ex. 1163, p. 77
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00006

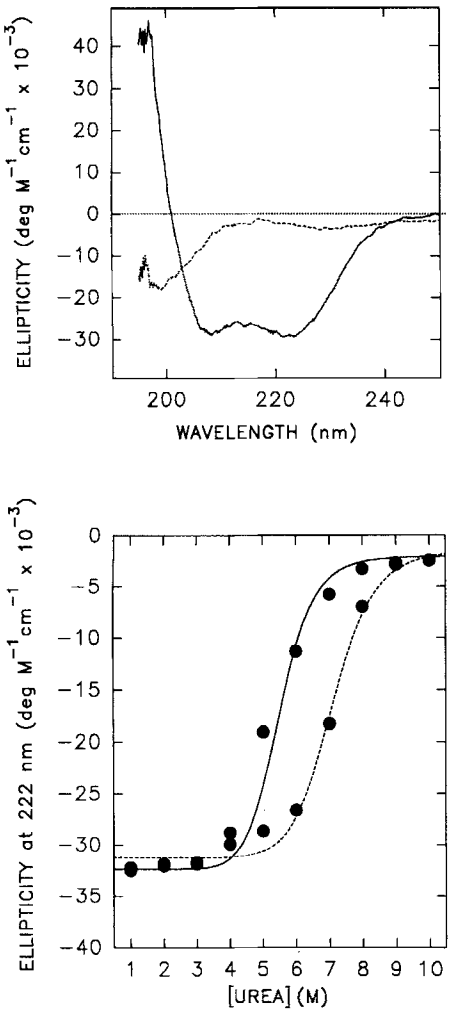


Fig. 3. CD spectra of peptides LZ and LZ7P14P and urea denaturation of peptides LZ and biotinyl-LZ. (A) CD spectra of peptide LZ (solid line) and peptide LZ7P14P (dashed line) in 8 mM phosphate, 0.14 M NaCl, pH 7.0, 20°C. (B) Urea denaturation at 20°C of peptide LZ (solid line) and peptide biotinyl-LZ (dashed line). CD spectra were measured with 20 μM peptide solutions.

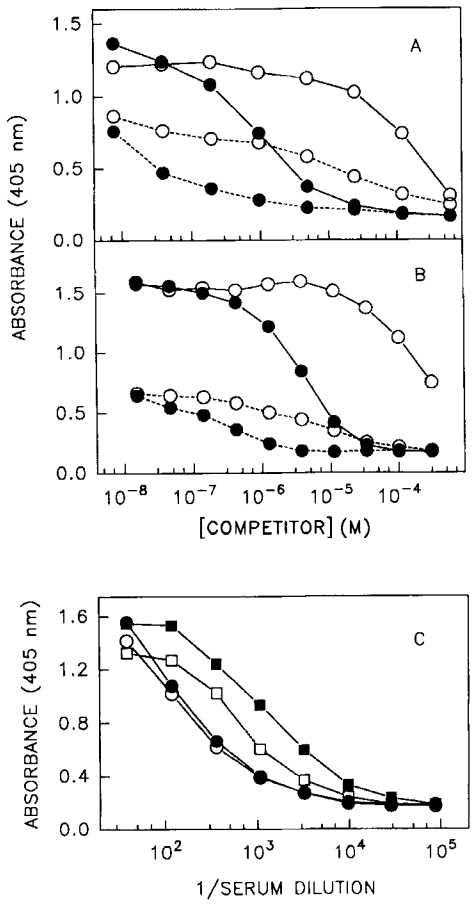


Fig. 4. Cross-reaction of antibodies to coiled-coil peptide LZ and random-coil peptide LZ7P14P. (A) Antiserum (1:40000) against biotinyl-LZ-disulfide assayed with biotinyl-LZ (cognate tracer antigen, solid lines) or biotinyl-LZ7P14P (non-cognate tracer antigen, dashed lines) in the presence of increasing amounts of peptide LZ (cognate competitor, ●) or peptide LZ7P14P (non-cognate competitor, ○). (B) Antiserum (1:200) against peptide LZ7P14P assayed with biotinyl-LZ7P14P (cognate tracer antigen, solid lines) or biotinyl-LZ (non-cognate tracer antigen, dashed lines) in the presence of increasing amounts of peptide LZ7P14P (cognate competitor, ●) or peptide LZ (non-cognate competitor, ○). In (A and B), the cross-reaction is genuine. Tracer concentration was 0.3 μM. (C) Solid-phase antibody-capture ELISA with antiserum to peptide LZ (○, ●) and to peptide LZ7P14P (□, ■). Antigen coated to the microtiter plate (0.1 μg/well) was peptide LZ (○, □) or peptide LZ7P14P (●, ■). Bound antibody was detected with anti-rabbit IgG-alkaline phosphatase conjugate and *p*-nitrophenylphosphate as substrate. Antibodies against peptide LZ do not distinguish against peptide LZ7P14P in this assay.

Competition immunoassays are shown in Fig. 4A for antibodies to the disulfide-linked peptide LZ. Using the cognate antigen biotinyl-LZ as the tracer, peptide LZ was a stronger competitor than peptide LZ7P14P (compare solid lines). Peptide LZ7P14P may be regarded the denatured form of peptide LZ. Peptide LZ was still a stronger competitor with the non-cognate tracer antigen biotinyl-LZ7P14P (compare dashed lines). These results are typical for a genuine cross-reaction with random-coil peptide LZ7P14P. The same results were obtained with antibodies from a rabbit immunized with peptide LZ that was not disulfide-linked (data not shown). In the experiment of Fig. 4A, the cognate antigen was a stronger competitor to the non-cognate tracer than to the cognate tracer. This is due to the low avidity of the genuinely cross-reacting antibody fraction for the non-cognate tracer; a lower concentration of cognate peptide LZ sufficed to displace antibodies from the non-cognate tracer.

Antibodies from rabbits immunized with peptide LZ7P14P again showed genuine cross-reaction. Peptide LZ7P14P was always the stronger competitor, independent of the tracer antigen used (Fig. 4B).

In a solid-phase antibody-capture ELISA, antibodies to peptide LZ could not distinguish between peptides LZ and LZ7P14P. Antibodies to peptide LZ7P14P were weakly discriminating in the same assay (Fig. 4C). This observation emphasizes that the assay format is critical to assess the conformational specificity of antibodies.

Antibodies to FNR peptides

The first eighteen residues of FNR are disordered (Karplus et al., 1991). Rabbit antiserum was produced against peptide biotinyl-FNR(1−20) bound to avidin. A fairly large fraction of this serum recognized native FNR. This follows

Petitioner Merck, Ex. 1163, p. 78
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00006

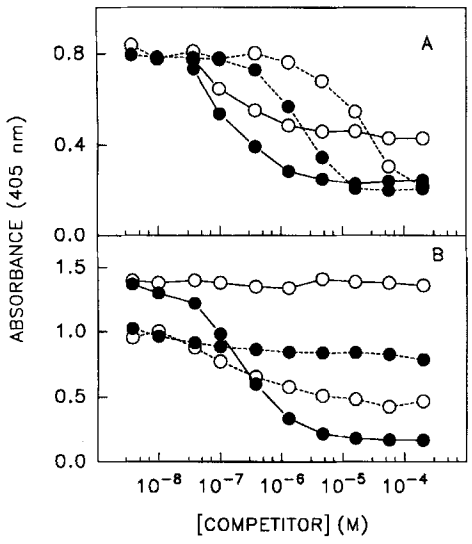


Fig. 5. Cross-reaction of antibodies to peptides FNR(1–20) and FNR(30–39) with native FNR. (A) IgG fraction of serum to FNR(1−20) assayed with biotinyl-FNR(1−20) (cognate tracer antigen, solid lines) or biotinylated FNR (non-cognate tracer antigen, dashed lines) in the presence of increasing amounts of FNR(1−20) (cognate competitor, ●) or native FNR (non-cognate competitor, ○). The cross-reaction is genuine. (B) IgG fraction of serum to FNR(30−39) assayed with biotinyl-FNR(30−39) (cognate tracer antigen, solid lines) or biotinylated FNR (non-cognate tracer antigen, dashed lines) in the presence of increasing amounts of FNR(30−39) (cognate competitor, ●) or native FNR (non-cognate competitor, ○). The cross-reaction is of the apparent type. IgG was 0.2 mg/ml and tracer 0.2 μM.

from the competition experiment shown in Fig. 5A in which the competition curve with FNR levels off at an absorbance value of about 0.5. Peptide FNR(1−20) was a stronger competitor for both tracer antigens, indicative of a genuine cross-reaction of many anti-peptide antibodies with native FNR.

Peptide FNR(30−39) corresponds to an exposed area of native FNR in which the sequence 33−36 forms a turn-like structure. Antiserum to FNR(30−39) recognized intact FNR in the solid-phase antibody-capture ELISA (data not shown). In this assay, FNR was coated directly to the microtiter plate and was probably unfolded. Hence this assay could not detect genuine cross-reaction with native FNR. Indeed, in the competition PACE in which biotinyl-FNR(30−39) was the tracer, FNR did not compete (Fig. 5B). Native FNR acted as a competitor when biotinylated FNR was the tracer antigen (Fig. 5B). FNR competed even better than free peptide FNR(30−39), (compare dashed lines in Fig. 5B).

DISCUSSION

The conformational specificty of protein-specific antibodies is well known and often utilized in biochemical applications. Perhaps less appreciated are the concepts of antibody cross-reactivity addressed here. On the one hand, the situation is uncomplicated for antibodies against a denatured protein. The cross-reaction is a genuine property of the antibodies provided that the immunoassay used to test cross-reactivity does not give erroneous results because the native antigen is unfolded during the assay. The problem is more complex with antibodies against a folded native protein where the cross-reaction may be either genuine or only apparent. Anti-protein antibodies are, in general, conformation-specific, consequently some investigators believe genuine cross-reaction with unfolded proteins or peptides is not possible (Jemmerson, 1987; Laver et al., 1990). The present results with anti-(cytochrome *c*) antibodies seem to support this view. Still, we cannot exclude the existence of antibodies to native cytochrome *c* that cross-react in a genuine way with the unfolded polypeptide chain. However, genuinely cross-reactive antibodies, if they existed, were concealed by a majority of apparently cross-reactive antibodies. The danger of unfolding, particularly in Freund's adjuvant, is substantial even in the case of a relatively stable protein like cytochrome *c*. Interestingly, apparently cross-reacting antibodies dominated when the monomeric protein was used for immunization (Fig. 1B), while the fraction of apparently cross-reacting antibodies was smaller for the glutaraldehyde-polymerized immunogen (Fig. 1A). Polymerization, and perhaps also conjugation to a carrier, may stabilize the folded structure and thereby suppress production of antibodies against the unfolded immunogen.

The high species-specificity of antibodies to horse cytochrome *c* was lost in the assay with the non-cognate antigen apo-cytochrome *c* (Fig. 2C). In support, antiserum AH1, raised against horse apo-cytochrome *c*, lacked specificity for the sequence differences between horse and rabbit cytochrome *c*. The majority of antigenic determinants recognized by serum AH1 were located within the first N-terminal third of the polypeptide chain, which has the same sequence in horse and rabbit cytochrome *c*. Immunological tolerance of the rabbit toward its own cytochrome c leads to clustered epitopes in areas where rabbit cytochrome *c* differs from related mammalian c-type cytochromes (Benjamin et al., 1984). Our results show that this tolerance is observed only in the context of the native protein fold.

Why did genuinely cross-reactive antibodies dominate in the case of the leucine-zipper peptide? We believe this is due to the high conformational stability of peptide LZ (Fig. 3B). Essentially no unfolded monomeric peptide LZ may have been presented to the immune system, therefore the cross-reactive antibodies were primarily directed to the coiled-coil form of the peptide. What is the molecular mechanism of the genuine cross-reaction of anti-LZ antibodies with peptide LZ7P14P? From the denaturation profile of peptide LZ shown in Fig. 3B we estimate a free energy of unfolding in water of 14−17 kJ/mol (analysis of the denaturation profile according to Pace, 1986). This conformational stability makes cross-reaction by induced fit unattractive if an antibody to peptide LZ has to force peptide LZ7P14P into a helical, coiled-coil-like structure in order to bind. It is more likely that cross-reacting antibodies were directed against epitopes common to both peptides. Such epitopes are of the sequential type and may occur in the more mobile N-terminal and C-terminal segments of peptide LZ. Cross-reaction with sequential epitopes is supported by the results from the solid-phase ELISA (Fig. 4C). Peptide LZ was at least partly unfolded on the microtiter plate and the cross-reactive antibodies belonged to the subpopulation directed against the sequential determinants that are common to peptides LZ and LZ7P14P.

The results with antisera to the FNR peptides provide different examples of protein reactivity. The strong protein reactivity of antibodies to peptide FNR(1−20) is not surprising since the N-terminal fragment of FNR is disordered and well accessible (Karplus et al., 1991). Mobility and easy accessibility is often seen at N-termini and C-termini of pro-

Petitioner Merck, Ex. 1163, p. 79
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00006

teins. Peptide FNR(30−39), although well exposed in native FNR, gave rise to very few cross-reactive antibodies. Most interestingly, these cross-reactive antibodies were only detected when using biotinylated FNR as the tracer antigen (Fig. 5B). Hence, the cross-reaction was of the apparent type according to our criterion. That is, the FNR-reactive antibodies were directed to a conformational isoform of peptide FNR(30−39). Peptide FNR(30−39) as used for immunization is probably a random coil. Among the many conformers in which the peptide was presented to B-cell receptors, a few may have been similar to the turn-like fold in the intact enzyme, giving rise to the few FNR-reactive antibodies. Incidentally, peptide 30−39 was predicted to be antigenic by the algorithm of Parker et al. (1986), (data not shown).

In conclusion, we have presented an experimental distinction between two types of cross-reaction by antisera to proteins and peptides. Since even a stable protein like cytochrome *c* unfolds to some degree during the complex events that lead to the production of soluble antibodies, apparently cross-reacting antibodies may often dominate the anti-protein response. Similarly, protein-reactive anti-peptide antibodies can be directed against a rare conformational isoform of the peptide used for immunization and the cross-reaction with the native protein is the property of only very few anti-peptide antibodies. Looked at in a different way, a limited degree of resemblance of the peptide conformation to the native protein may suffice to produce some protein-reactive antibodies. Attempts to improve the yield of protein-reactive antibodies by restricting the flexibility of synthetic peptides, thereby improving conformational mimicry, has had some success (Dyson et al., 1988; Saragovi et al., 1992; Joisson et al., 1993).

The different concepts of cross-reactivity should be considered when using antisera as conformation-specific probes. Only by selecting an approriate solution-phase immunoassay can one distinguish between apparent and genuine antibody cross-reaction. Finally, one may possibly control to some extent the amount and type of cross-reacting antibodies by varying the type of cross-linking or polymerization of the immunogen.

We thank Dr Peter Hunziker for analyses of synthetic peptides by ion-spray mass spectrometry. This work was supported in part by the Swiss National Science Foundation, the *Stiftung für wissenschaftliche Forschung der Universität Zürich*, and by the *Hartmann Müller-Stiftung*.

REFERENCES

Anderer, F. A. (1963) Preparation and properties of an artificial antigen immunologically related to tobacco mosaic virus, *Biochim. Biophys. Acta 71*, 246−248.

Arnon, R. (1987) *Synthetic vaccines*, CRC Press, Boca Raton FL.

Arnon, R. & Van Regenmortel, M. H. V. (1992) Structural basis of antigenic specificity and design of new vaccines, *FASEB J. 6*, 3265−3274.

Benjamin, D. C., Berzofsky, J. A., East, I. J., Gurd, F. R. N., Hannum, C., Leach, S. J., Margoliash, E., Michael, J. G., Miller, A., Prager, E. M., Reichlin, M., Sercarz, E., Smith-Gill, S. J., Todd, P. E. & Wilson, A. C. (1984) The antigenic structure of proteins, a reappraisal, *Annu. Rev. Immunol. 2*, 67−101.

Berzofsky, J. A. & Schechter, A. N. (1981) The concepts of cross-reactivity and specificity in immunology, *Mol. Immunol. 18*, 751−763.

Brawn, R. J. & Dandliker, W. B. (1977) The effect of antigen structure on preferential humoral or cellular immunogenicity, In *Immunochemistry of proteins* (Atassi, M. Z., ed.) vol. 2, pp. 45−76, Plenum Press, New York.

Chen, Y., Yang, Y. T. & Martinez, H. M. (1972) Determination of the secondary structure of proteins by circular dichroism and optical rotatory dispersion, *Biochemistry 11*, 4120−4131.

Damaschun, G., Damaschun, H., Gast, K., Gernat, C. & Zirwer, D. (1991) Acid denatured apo-cytochrome *c* is a random coil: Evidence from small-angle X-ray scattering and dynamic light scattering, *Biochim. Biophys. Acta 1078*, 289−295.

Dyson, H. J., Lerner, R. A. & Wright, P. E. (1988) The physical basis for induction of protein-reactive antipeptide antibodies, *Annu. Rev. Biophys. Biophys. Chem. 17*, 305−324.

Friguet, B., Djavadi-Ohaniance, L. & Goldberg, M. E. (1984) Some monoclonal antibodies raised with a native protein bind preferentially to the denatured antigen, *Mol. Immunol. 21*, 673−677.

Hodges, R. S., Semchuk, P. D., Taneja, A. K., Kay, C. M., Parker, J. M. R. & Mant, C. T. (1988) Protein design using model synthetic peptides, *Pept. Res. 1*, 19−30.

Jemmerson, R. (1987) Antigenicity and native structure of globular proteins: Low frequency of peptide reactive antibodies, *Proc. Natl Acad. Sci. USA 84*, 9180−9184.

Jemmerson, R. & Margoliash, E. (1979) Topographic antigenic determinants on cytochrome *c*. Immunoabsorbent separation of the rabbit antibody populations against horse cytochrome *c*, *J. Biol. Chem. 254*, 12706−12716.

Joisson, C., Kuster, F., Plaué, S. & Van Regenmortel, M. H. V. (1993) Antigenic analysis of bean pod mottle virus using linear and cyclized synthetic peptides, *Arch. Virol. 128*, 299−317.

Karplus, P. A., Daniels, M. J. & Herriott, J. R. (1991) Atomic structure of ferredoxin-$NADP^+$ reductase: prototype for a structurally novel flavoenzyme family, *Science 251*, 60−66.

Kuo, L. M. & Davies, H. C. (1983) Production, isolation and characterization of monoclonal antibodies to cytochrome *c* of beef heart and *P. denitrificans*, *Mol. Immunol. 20*, 827−838.

Landsteiner, K. (1945) *The specificity of serological reactions*, Harvard University Press, Cambridge, Mass.

Laver, W. G., Air, G. M., Webster, R. G. & Smith-Gill, S. J. (1990) Epitopes on protein antigens: Misconceptions and realities, *Cell 61*, 553−556.

Lovejoy, B., Choe, S., Cascio, D., McRorie, D. K., DeGrado, W. F. & Eisenberg, D. (1993) Crystal structure of a synthetic alpha-helical bundle, *Science 259*, 1288−1293.

Michel, B., Proudfoot, A. E. I., Wallace, C. J. A. & Bosshard, H. R. (1989) The cytochrome *c* oxidase − cytochrome *c* complex: Spectroscopic analysis of conformational changes in the protein-protein interaction domain, *Biochemistry 28*, 456−462.

Ngai, P. K., Ackermann, F., Wendt, H., Savoca, R. & Bosshard, H. R. (1993) Protein A antibody-capture ELISA (PACE): An ELISA format to avoid denaturation of surface-adsorbed antigens, *J. Immunol. Methods 158*, 267−276.

O'Neil, K. T. & DeGrado, W. F. (1990) A thermodynamic scale for the helix-forming tendencies of the commonly occurring amino acids, *Science 250*, 646−651.

Pace, C. N. (1986) Determination and analysis of urea and guanidine hydrochloride denaturation curves, *Methods Enzymol. 131*, 266−280.

Parker, J. M. R., Guo, D. & Hodges, R. S. (1986) New hydrophilicity scale derived from high-performance liquid chromatography peptide retention data: correlation of predicted surface residues with antigenicity and x-ray-derived accessible sites, *Biochemistry 25*, 5425−5432.

Pellequer, J. L., Westhof, E. & Van Regenmortel, M. H. V. (1991) Predicting location of continuous epitopes in proteins from their primary structures, *Methods Enzymol. 203*, 176−201.

Reichlin, M., Nisonoff, A. & Margoliash, E. (1970) Immunological activity of cytochrome *c*. III. Enhancement of antibody detection and immune response initiation by cytochrome *c* polymers, *J. Biol. Chem. 245*, 947−954.

Saad, B., Corradin, G. & Bosshard, H. R. (1988) Monoclonal antibody recognizes a conformational epitope in a random coil protein, *Eur. J. Biochem. 178*, 219−224.

Saragovi, H. U., Greene, M. I., Chrusciel, R. A. & Kahn, M. (1992) Loops and secondary structure mimetics: Development and

Petitioner Merck, Ex. 1163, p. 80
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00006

applications in basic science and rational drug design, *Biotechnology (NY) 10*, 773–778.
Savoca, R., Schwab, C. & Bosshard, H. R. (1991) Epitope mapping employing immobilized synthetic peptides: How specific is the reactivity of these peptides with antiserum raised against the parent protein? *J. Immunol. Methods 141*, 245–252.
Scholtz, J. M. & Baldwin, R. L. (1992) The mechanism of alpha-helix formation by peptides, *Annu. Rev. Biophys. Biomol. Struct. 21*, 95–118.
Schwab, C., Twardek, A., Lo, T. P., Brayer, G. D. & Bosshard, H. R. (1993) Mapping antibody binding sites with synthetic peptides: Are results representative of the antigenic structure of proteins? *Protein Sci. 2*, 175–182.
Schwab, C. & Bosshard, H. R. (1992) Caveats for the use of surface-adsorbed protein antigen to test the specificity of antibodies, *J. Immunol. Methods 147*, 125–134.
Scibienski, R. J. (1973) Denaturation of lysozyme by Freund's complete adjuvant, *J. Immunol. 111*, 114–120.
Scott, D., Nitecki, D. E., Kindler, H. & Goodman, J. W. (1984) Immunogenicity of biotinylated hapten-avidin complexes, *Mol. Immunol. 21*, 1055–1060.
Shin, M. & Oshino, R. (1978) Ferredoxin-Sepharose 4B as a tool for the purification of ferredoxin-$NADP^+$ reductase, *J. Biochem. (Tokyo) 83*, 457–461.
Spangler, B. D. (1991) Binding to native proteins by antipeptide monoclonal antibodies, *J. Immunol. 146*, 1591–1595.
Talmadge, D. (1959) Immunological specificity, *Science 129*, 1643–1648.
Urbanski, G. J. & Margoliash, E. (1977) Topographic determinants on cytochrome *c*, *J. Immunol. 118*, 1170–1180.
Van Regenmortel, M. H. V. (1989) The concept and operational definition of protein epitopes, *Philos. Trans. R. Soc. Lond. B Biol. Sci. 323*, 451–466.