

Thiol Reactivity of the Nitrogenase Fe-Protein from *Azotobacter vinelandii**

(Received for publication, April 8, 1983)

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A procedure has been developed to examine some of the functional roles of the 14 cysteinyl residues in the nitrogenase Fe-protein (Av2) from *Azotobacter vinelandii*. The reduced form of Av2 was alkylated with iodo[2-¹⁴C]acetic acid under a variety of experimental conditions, e.g. reaction in the presence of nucleotides, α,α' -dipyridyl and nucleotides, or denaturants. The labeled cysteinyl residues were identified and quantified using an analytical DEAE-Sepharose ion exchange chromatography peptide mapping technique based upon the known amino acid sequence (Hausinger, R. P., and Howard, J. B. (1982) *J. Biol. Chem.* 257, 2483-2490). From the results of the labeling experiments, the following features of the Av2 structure have been proposed. 1) Av2 contains no disulfides, hyper-reactive thiols, or surface thiols as defined by reaction with iodoacetic acid. 2) Cysteines 97 and 132 are the probable ligands for the Av2 Fe:S center which is bound symmetrically between subunits. 3) MgATP partially protects cysteine 85 from carboxymethylation by iodoacetic acid and may be part of the nucleotide-binding site. 4) Of the five nonligand thiols only cysteines 5 and 184 are completely alkylated when Av2 is denatured in hexamethylphosphoramide, whereas all five nonligand thiols appear to rapidly exchange at the Fe:S center if the protein is denatured in the absence of alkylating reagents. 5) Both Av2 and apo-Av2 appear to undergo a reversible conformational change upon binding MgATP.

Biological reduction of dinitrogen to ammonia is catalyzed by a complex of two enzymes, the MoFe-protein and the Fe-protein. The MoFe-protein contains the substrate-binding site while reduced Fe-protein serves as the electron donor for the system (1). Electron transfer and substrate reduction require MgATP hydrolysis and are inhibited by MgADP or Mg₂ATP (1). The Fe-protein dimer has two MgATP-binding sites (presumably 1 site per subunit); however, both enzyme components are needed for ATP hydrolysis (1).

The amino acid sequence of the Fe-protein subunit (*M*_r = 29,000-34,000) from five different species has been determined either directly by protein structural analysis (2, 3) or by deduction from the DNA sequence (4-6). These sequences are ~60% identical with the amino acid substitutions limited

to a few regions. Cysteinyll residues are found at 10 different positions in the sequence, 5 of which are conserved (see Table I). The Fe-protein contains a single 4Fe:4S cluster that has been proposed to bridge the identical subunits (7). Upon binding MgATP, the protein undergoes a conformational change that results in altered properties of the Fe:S center. For example, the *E'*_o (pH 7.5) decreases ~100 mV (8), the EPR spectra becomes more axial (9), and the magnetic circular dichroic spectrum changes (10). Perhaps, the most dramatic effect of MgATP binding is the change in reactivity of the Fe:S center with chelators. Walker and Mortenson (11) and Ljones and Burris (12) found that the Fe:S center was rapidly decomposed by chelators in the presence of MgATP but not with MgADP or in the absence of nucleotide. The amount of MgATP-dependent Fe chelation corresponded to the enzyme activity and was advocated as an assay for active Fe:S centers (12).

By analogy to other Fe:S proteins, the Fe-protein ligands are assumed to be cysteinyl residues. Unlike ferredoxins, the Fe-protein has more cysteines than the 4 required for Fe:S ligands. The additional residues may have roles such as nucleophiles in catalysis, redox centers in electron transfer, or structural cross-links as disulfides. Thorneley and Eady (13) investigated the chemical reactivity of Kp2' thiols with DTNB and found that 3 times as many equivalents of DTNB reacted with the protein in the presence of MgATP as in its absence. However, they did not distinguish between DTNB reaction with sulfide (2 DTNB) and cysteine (1 DTNB) (13). In contrast, Mortenson *et al.* (14) found that the number of Cp2 cysteines carboxymethylated by IAA decreased by 2 in the presence of MgATP. The degree of modification and the identity of the residues modified were not reported (14).

In this paper, we report studies on the functional roles of the Av2 cysteinyl residues using the chemical reactivity of IAA with the thiols as a probe. We have attempted to determine: does Av2 have disulfides; are there exposed, chemically reactive cysteines; do the cysteines have different relative chemical reactivity upon nucleotide binding; and which cysteines are the ligands of the 4Fe:4S center.

MATERIALS AND METHODS

Iodo[2-¹⁴C]acetic acid and iodo[2-³H]acetic acid were obtained from New England Nuclear. Stock solutions of IAA were prepared by diluting the radioactive reagents with recrystallized unlabeled IAA in water and adjusting the solution to pH 6-7 with NaOH. To determine the specific radioactivity of the stock solutions, IAA was incubated with excess cysteine at pH 8.0 for 1 h and duplicate samples were run on the amino acid analyzer. One sample was analyzed for carboxymethylcysteine content by ninhydrin; the other sample was analyzed

¹ The abbreviations used are: Av2, Cp2, and Kp2 for the Fe-protein from *Azotobacter vinelandii*, *Clostridium pasteurianum* and *Klebsiella pneumoniae*, respectively; [¹⁴C]IAA, iodo[2-¹⁴C]acetic acid; [³H]IAA, iodo[2-³H]acetic acid; IAA, iodoacetic acid; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

* This work was supported by Grant 5901-0410-80106 from the Science and Education Administration of the United States Department of Agriculture. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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TABLE I
Distribution of cysteinyl residues in the Fe-protein from different species

+ indicates that a cysteine is found at this residue position in the sequence (numbering is based upon Av-2 (3)).

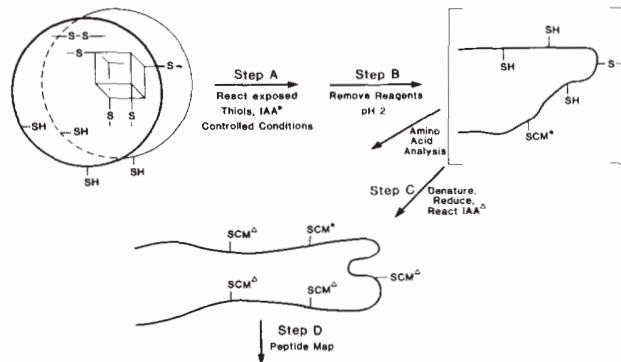
Source	5	38	85	97	132	151	184	234	259	296	Total cysteines	Total residues
<i>Azotobacter vinelandii</i> (3)	+	+	+	+	+	+	+				7	289
<i>Klebsiella pneumoniae</i> (5)	+	+	+	+	+	+	+	+	+		9	292
<i>Clostridium pasteurianum</i> (2)		+	+	+	+		+	+			6	273
<i>Anabaena</i> (4)		+	+	+	+		+			+	6	299
<i>Rhizobium meliloti</i> (60)		+	+	+	+		+				5	297

for radioactivity by scintillation counting of the column eluant before ninhydrin reaction. The specific radioactivity of the stock solutions varied from 200–2500 cpm per nmol of IAA. The choice of stock solution used depended upon the requirements of the experiment.

The Fe-protein and the MoFe-protein from *Azotobacter vinelandii* (ATCC 13705) were prepared using a modified procedure of Burgess *et al.* (15). Protein concentrations were determined by amino acid analysis. Methods of peptide isolation, amino acid sequencing, and scintillation counting are described in our earlier communications (3, 16, 17). Ultraviolet-visible spectra and continuous single wavelength monitoring of protein samples were recorded with a Beckman DU-8 spectrophotometer using a double chamber anaerobic cuvette similar to that described by Averill *et al.* (18). Solutions were transferred using gas-tight syringes. Fe released from the protein under various experimental conditions was determined by its complex with α, α' -dipyridyl ($\epsilon_{520\text{nm}} = 8.4 \text{ cm}^{-1} \text{ mM}^{-1}$ (11)).

Radiolabeled cysteinyl residues were identified and quantified using a modification of our ion exchange peptide map for Av2 tryptic peptides (3, 19). Av2, uniformly labeled by exhaustive reductive carboxymethylation with [^3H]IAA, was added to Av2 selectivity modified with [^{14}C]IAA (see under "Results and Discussion" for specific experiments). On the basis of radioactivity, approximately equal amounts of protein (0.5–3.0 mg, $1\text{--}2 \times 10^5$ cpm each) were combined and digested with tosylphenylalanyl chloromethyl ketone-treated trypsin (2% added initially, 2% added after 2 h) for 4 h at 25 °C in 0.5 ml of pH 8.0, 0.1 M Tris·HCl buffer. A portion of the peptide solution ($\sim 10^5$ cpm) was applied to a column (1 × 50 cm) of DEAE-Sephacel equilibrated with pH 8.0, 0.1 M Tris·HCl buffer. The peptides were eluted with a 200-ml linear NaCl gradient (70 mM–1.0 M) at 20 °C, final concentration). Two-ml fractions were collected and the radioactivity in 1 ml was determined by multichannel scintillation counting using automatic quench correction. To determine the amount of ^{14}C and ^3H in each fraction, the ^3H values were corrected for 22% overlap from the ^{14}C counting channel (determined experimentally). The 6 tryptic peptides containing 7 cysteinyl residues are readily separated on this map (see Fig. 2 for a sample chromatogram). The order of elution of the peptides has been correlated to their location in the complete amino acid sequence, *viz.* the peptides elute in the order cysteine –184, –5, –38, –151, –85 and –97 (a dicysteinyl peptide), and –132 (3). The peptide-containing residue 151 eluted primarily as the 4th radioactive peptide but also as two smaller peaks after the dicysteinyl peptide (see Fig. 2). Based upon the average of 37 different peptide maps from several different protein preparations, the recovery of radioactivity in these peaks was $84 \pm 6\%$ ($\pm \text{S.D.}$) of that applied to the column. The ratios of the primary peaks from the uniformly labeled [^3H]protein were 1.00:0.95:1.03:0.41:2.01:0.89. The ratio for cysteine 151 was increased from 0.41 to 0.83 when the two minor peaks were included. No peak varied more than 10% from the established ratio. That each radioactive peak contained only one radioactive peptide was established earlier and confirmed by high performance liquid chromatography (3).

To distinguish between cysteines 85 and 97, the radioactive peak corresponding to the dicysteinyl peptide was pooled and desalted on a column (1 × 25 cm) of Sephadex G-25 in pH 9.0, 10 mM NH_4 acetate. The peptide was lyophilized, dissolved in 0.2 ml of pH 8.0, 10 mM Tris·HCl buffer, and digested with 10% (by weight) thermolysin for 2 h at 37 °C. The peptides were separated by high performance liquid chromatography on a column (0.46 × 15 cm) of C-18 matrix (Altex, 10- μ particle), using a linear 50-min gradient from 0.1% F_3COOH to 45% acetonitrile/0.1% F_3COOH at a flow rate of 1.0 ml per min. One-min fractions were collected and the radioactivity counted (see Fig. 2, inset). The first radioactive peak corresponds to residues 95–97 (including cysteine 97), the second peak is residues 85–94 (including cysteine 85), and the final peak is undi-



SCHEME 1. Outline of experimental protocol for labeling of selected cysteinyl residues. See text for details of methods. * and Δ are radioactive and unlabeled iodoacetic acid where the order of use is not specified. CM-, carboxymethyl derivative.

gested peptide, residues 85–100. The latter provided an additional check on the $^{14}\text{C}/^3\text{H}$ ratio observed in the DEAE-Sephacel column map.

EXPERIMENTAL PROTOCOL

To ascertain the functional role of the cysteinyl residues in Av2, an experimental protocol was developed whereby specific cysteines could be labeled under controlled conditions. The protocol is outlined in Scheme 1.

Step A—Deaerated buffer (pH 8.0, 0.1 M Tris·HCl containing 2 mM $\text{Na}_2\text{S}_2\text{O}_4$) and reagents to perturb the protein (see under "Results and Discussion" for specific experiments) were added to an anaerobic cuvet. If cysteines were to be labeled, [^{14}C]IAA was included. Alternatively, unlabeled reagents were used and the unreacted cysteines labeled at a later step (Step C). To initiate the reaction, Av2 (60–100 nmol) in pH 8.0, 0.1 M Tris·HCl, 0.5 M NaCl buffer containing 2 mM $\text{Na}_2\text{S}_2\text{O}_4$ was added. The reaction was continuously monitored in the spectrophotometer. The stability of the Fe:S center was monitored at 410 nm or the complex of α, α' -dipyridyl with Fe released from the protein was observed at 520 nm. At specified times of incubation, the reaction was stopped by the addition of 0.25 ml of glacial acetic acid.

Step B—Excess reagents were removed by exhaustive dialysis, in turn, against 10% acetic acid, water, and 10 mM NH_4 acetate. The fraction of cysteines carboxymethylated was determined by amino acid analysis.

Step C—The lyophilized protein was fully carboxymethylated in 6 M guanidine HCl. Either unlabeled IAA or [^{14}C]IAA was used to complement the reagent used in Step A. The reagents were removed by dialysis as indicated in Step B.

Step D—The specifically labeled protein was combined with uniformly ^3H -labeled protein and a tryptic peptide map was prepared as described under "Materials and Methods." From the ratio of ^{14}C to ^3H for each peak in the map and the fraction of cysteines carboxymethylated during the radiolabeling step, the molar fraction of any specific cysteine labeled could be

calculated by the following formula,

$$\text{Mole fraction of residue modified} = \frac{(^{14}\text{C}/^3\text{H})_{\text{residue}}}{(^{14}\text{C}/^3\text{H})_{\text{average}}} \times \text{fraction of all cysteines modified during radiolabeling step.} \quad (1)$$

On the basis of amino acid analysis, <5% of any amino acid other than cysteine was labeled using this protocol.

RESULTS AND DISCUSSION

Does Av2 Contain Disulfides?—Although small electron transfer Fe:S proteins, *e.g.* ferredoxins, do not contain disulfides, larger Fe:S enzymes could have disulfides as well as free cysteines and cysteinyl ligands to the Fe:S center. The protocol for determining the latter classes of cysteines requires knowledge of the disulfide content of the protein. To determine the number of disulfides, the Fe:S center must be decomposed under reaction conditions that do not generate blocked cysteines, *e.g.* disulfides. Furthermore, disulfides that might be in the protein must not be reduced by the reagents. Two methods based upon the outline in Scheme 1 have been used successfully to demonstrate that Av2 does not contain disulfides.

First, in Scheme 1, *Step A*, 67 nmol of Av2 were added to a pH 8.0, 0.1 M Tris·HCl, 2 mM Na₂S₂O₄ buffer containing at final concentration, 3.4 M guanidine HCl, 5.6 mM EDTA, and 83 mM IAA. The protein solution was colorless within 30 s. After 30 min, the reaction was stopped and the reagents removed (Scheme 1, *Step B*). In *Step C*, [¹⁴C]IAA was used to quantify and identify the cysteines unreacted in *Step A*.

Because the Fe:S centers in many ferredoxins² are partially stable under the conditions of the protocol above, a second approach was tested. In *Step A*, the protein was added to a guanidine HCl solution containing EDTA and IAA at pH 2.0. Within 30 s the brown solution turned colorless and 2 M Tris base was introduced to raise the pH. The final reaction conditions, *Step B*, and *Step C* were the same as for the first method.

The results for both methods were >95% carboxymethylation in *Step A* (by amino acid analysis) and <5% carboxymethylation in *Step C* (by incorporation of radioactivity). If Av2 were to contain a single disulfide, 86 and 14% carboxymethylation, respectively, would have been expected for the two steps. To determine whether disulfides in Av2 had been reduced in *Step 2*, lysozyme (4 disulfides) was included in the reaction. In this control experiment, less than 2% carboxymethylation of lysozyme was found in *Step A* indicating little thiol-disulfide exchange between proteins or reduction of existing disulfides had occurred.

Does Av2 Have Surface Thiols?—Of the 14 cysteinyl residues per Av2 dimer, four are presumed to be ligands of the Fe:S center, and none are as disulfides. Thus, 10 cysteinyl residues can be described as “exposed” or “buried” thiols in terms of their chemical reactivity to various reagents. To determine if there are cysteinyl residues in native Av2 that are reactive with IAA, [¹⁴C]IAA was incubated with Av2 in pH 8.0, 0.1 M Tris·HCl and the absorbance at 410 nm recorded (Scheme 1, *Step A*). As shown in Fig. 1, there was a continuous decrease in absorbance indicating destruction of the Fe:S center. Although iron sulfide from the cluster interfered with the measurements and precluded the calculation of rate constants, a 65% absorbance loss was extrapolated for 120 min of reaction. For comparison, Av2 incubated in buffer without IAA had 5% absorbance change and no loss of enzyme activity in 120 min. Samples at specified time points were quenched with acetic acid and processed as outlined in

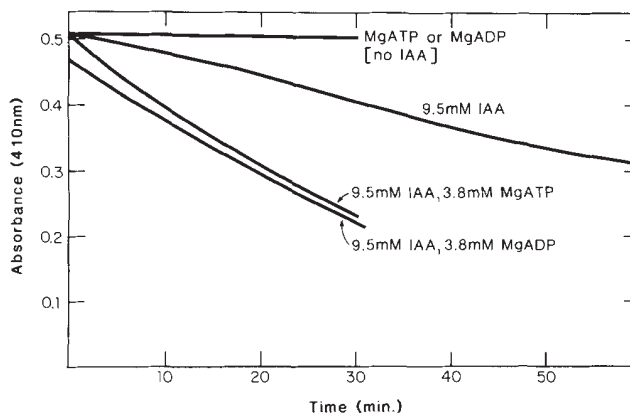


FIG. 1. Change in absorbance for Av2 chromophore (410 nm). Fe-protein (51.2 μ M) was anaerobically incubated with the indicated compounds in 0.1 M Tris·HCl, pH 8.0, buffer containing 2 mM Na₂S₂O₄ at 22 °C and the change at 410 nm recorded.

Scheme 1, *Steps B–D*. A sample peptide map for one time point (120 min) is shown in Fig. 2. The amount of carboxymethylation for each cysteine is given in Table II, A, for three representative time points. The pattern of modification indicates that no cysteine was rapidly alkylated, *i.e.* no cysteine is considered a surface residue by our criterion of IAA reaction. For example, less than 50% of any residue is labeled in 30 min; yet, as shown below, cysteines exposed in denaturant are completely alkylated in 5–10 min. Residues 85, 97, and 132 were alkylated 2- to 3-fold more than the others but only to a degree generally related to the loss of the Fe:S center. A more detailed correlation of absorbance change and carboxylation is not possible because of the interference from the decomposed Fe:S center and the uncertainty in extinction coefficient for a Fe:S cluster with one or more ligands modified. Mortenson *et al.* (14) found a similar total number of residues in Cp2 modified by IAA (3.7/12 residues in 10 min). However, they did not follow destruction of the Fe:S center or identify the residues modified.

Because the alkylation by IAA may be dependent on the ionic environment of the cysteine (20, 21), the reaction was repeated using the uncharged reagent, iodoacetamide (Scheme 1, *Step A*). The results (data not shown) were consistent with those shown in Table II.

Does Nucleotide Binding of Av2 Alter Cysteinyl Reactivity?—The binding of MgATP and MgADP by Fe-protein has been reported to induce substantial conformational changes in the protein (1, 8–14). To assess the change in cysteinyl reactivity, Av2 was incubated with [¹⁴C]IAA in the presence of MgATP or MgADP. The specific labeling of the cysteines was determined by the protocol outlined in Scheme 1 and the results are presented in Table II, B and C. As shown in Fig. 1, both nucleotides increased the apparent rate of IAA-dependent Fe:S center destruction. However, there was only a small increase in total carboxymethylation compared to the reaction without nucleotides. Rather, the main difference was the increased selectivity of the labeling toward residues 97 and 132 in the presence of nucleotide. The labeling of these residues was parallel to the absorbance loss of the Fe:S center. Residue 85 appears to be modified 30% less in the presence of MgATP than with MgADP or in buffer. The decreased reactivity could be the result of either an ATP-dependent allosteric “burying” of residue 85 or steric protection of a thiol in the nucleotide-binding site.

Our results are somewhat at variance with those presented by others (14, 15). Whereas Mortenson *et al.* (14) reported a 50% decrease in the total carboxymethylation of Cp2 in the

² J. B. Howard, T. Lorschach, and G. Anderson, unpublished results.

TABLE II
Carboxymethylation of Av2 by [^{14} C]IAA in pH 8.0, Tris·HCl buffer at 22 °C

At the time points, the reaction was stopped and peptide maps were generated as described in the text and shown in Fig. 2. The fraction of labeling for each residue was calculated using Equation 1. A, Av2 in buffer; B, Av2 in buffer with 3.8 mM MgATP; C, Av2 in buffer with 3.8 mM MgADP.

Reaction time	Protein residue number							% Carboxy-methylation	% A ₄₁₀ change ^a
	5	38	85	97	132	151	184		
<i>min</i>	<i>mol modified/mol of subunit</i>								
A. Av2 buffer									
5	0.16	0.17	0.10	0.11	0.21	0.18	0.20	16.1	6
30	0.17	0.21	0.37	0.49	0.43	0.05	0.20	27.4	24
120	0.43	0.41	0.65	0.80	0.63	0.18	0.43	50.4	65
B. Av2 + MgATP									
6	0.16	0.16	0.10	0.17	0.23	0.15	0.19	16.6	16
10	0.17	0.16	0.13	0.24	0.27	0.22	0.20	19.9	26
30	0.15	0.20	0.27	0.51	0.53	0.15	0.16	28.3	55
C. Av2 + MgADP									
5	0.13	0.13	0.10	0.29	0.25	0.16	0.17	17.6	9
10	0.18	0.16	0.16	0.41	0.37	0.15	0.18	23.0	29
30	0.15	0.24	0.39	0.52	0.55	0.21	0.20	32.3	52

^a Value is estimated. Fe₂S₂ released from Fe:S center of protein contributes to measured absorbance.

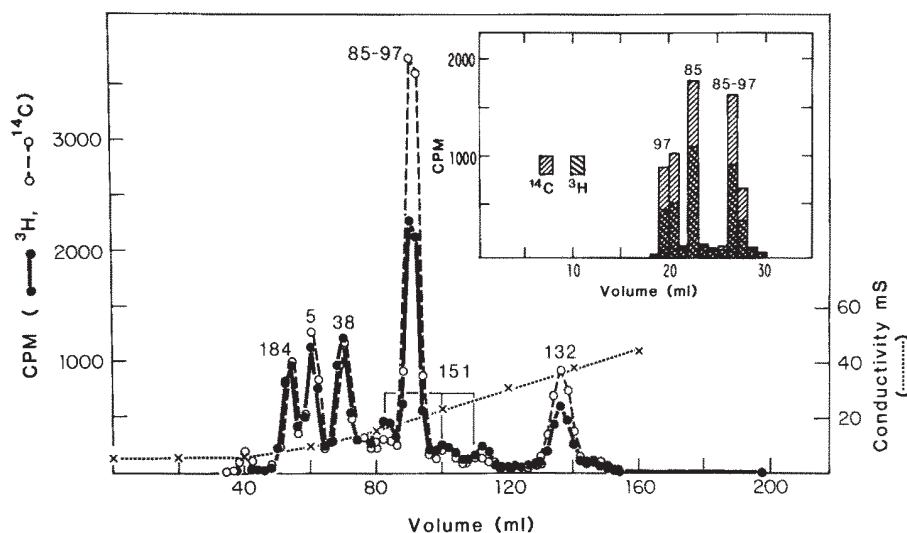


FIG. 2. Peptide column map for 120-min reaction of Av2 with [^{14} C]IAA in pH 8.0 buffer (see Fig. 1). Av2 labeled with [^{14} C]IAA was mixed with Av2 uniformly labeled by [^{3} H]IAA (5×10^5 cpm, each) and the protein digested with trypsin (see Materials and Methods). The peptides were separated by chromatography on DEAE-Sephacel (1×50 cm column) in pH 8.0 0.1 M Tris·HCl buffer using a linear 200-ml gradient to 70 mM NaCl. Half of each fraction was counted for radioactivity and analyzed as described in the text. The pooled dicycystinyl peptide was digested with thermolysin and the fragments were separated by high performance liquid chromatography on a C-18 matrix (0.46×15 cm column) using a 50-min linear gradient to 45% acetonitrile in 0.1% F₃CCOOH (see inset). Peaks are labeled according to the amino acid sequence numbering for the cysteinyl residue within the peptide at that position.

presence of MgATP, we find little change in the overall level of modification in the presence or absence of MgATP. Their findings are difficult to assess because they did not identify the residues modified. Furthermore, Cp2 has a cysteine at position 234 (alanine in Av2, see Table I) which might partially account for the difference. In contrast to both our work and that of Mortenson *et al.* (14), Thorneley and Eady (13) reported for Kp2 an increased thiol reactivity with DTNB in the presence of MgADP (35%) or MgATP (250%). Because Kp2 has 4 additional cysteines per dimer (see Table I), part of the increased reactivity may be due to these residues. A more likely explanation is that DTNB caused the decomposition of the Fe:S center and reacted with the newly released thiols and sulfide. Indeed, for each Fe:S center destroyed, 12 eq of DTNB can react. The specificity of the modified cysteines was not reported (13).

Which Cysteines in Av2 Are Exposed by Fe Chelators?—The

MgATP-dependent chelation of the Fe:S center provides an approach to specifically label the thiol ligands (11, 12). When our preparations of Av2 were added to a buffer containing α, α' -dipyridyl, there was no increase in absorbance at 520 nm indicating no readily available Fe (see Fig. 3). In the presence of MgATP and chelator, 4.3 mol of Fe per mol of protein were rapidly released while MgADP had no effect. These results are similar to those reported by others (11, 12). As shown in Fig. 3, IAA increased the Fe:S center destruction in the presence of MgATP and chelator. Furthermore, if IAA was included with either MgADP and chelator or chelator alone, the Fe rapidly became accessible to the chelator.

To identify the cysteinyl residues exposed by chelation of the Fe:S center, Av2 was incubated with α, α' -dipyridyl, [^{14} C] IAA, and either MgATP or MgADP. At specified times, the reaction was terminated and the protein was processed as described under "Experimental Protocol" (Steps B–D). The

patterns of labeling are reported in Table III for MgATP and MgADP. When Av2 was incubated with MgATP and α,α' -dipyridyl the total carboxymethylation at 30 min was $\sim 40\%$ greater than without the chelator. More important, the labeling of specific residues was greatly enhanced. For example, residues 97 and 132 were nearly completely modified after 10 min while these residues are only 20% modified (slightly more than other cysteines) if the chelator is not included (*cf.* Table II). The modification of residues 97 and 132 approaches that of a residue exposed in denaturant (see below). When MgADP and chelator were incubated with Av2, residue 85 and, to a lesser degree, residue 38, were labeled in addition to cysteines 97 and 132. The apparent protection of residue 85 by MgATP is similar to the results without chelator.

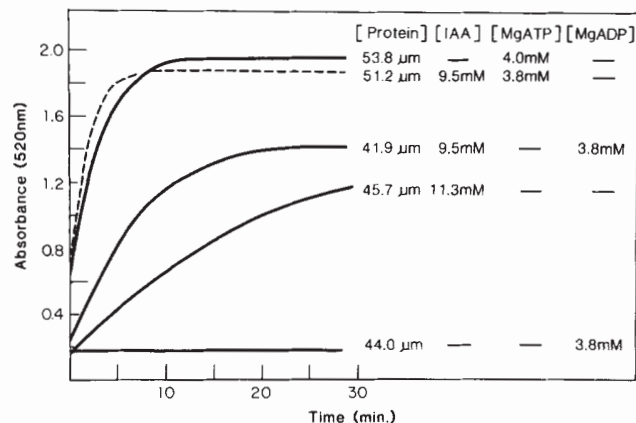


FIG. 3. Fe chelation of Av2 by α,α' -dipyridyl as monitored at 520 nm. Av2 was anaerobically incubated with 3.4 mM α,α' -dipyridyl in pH 8.0 0.1 M Tris·HCl buffer containing 2 mM $\text{Na}_2\text{S}_2\text{O}_4$ at 22 °C. Concentrations of Av2, [^{14}C]IAA, MgATP, or MgADP in the reaction are given in the figure.

The simplest interpretation of these results is that residues 97 and 132 are the thiol ligands of the Fe:S center. Alternatively, the reactive residues could be exposed by a conformational change following removal of the center and the true ligands buried as disulfides or trisulfides (22). To eliminate the latter possibility, Av2 was analyzed for disulfides (see section above for methods) after the MgATP-dependent chelation of the Fe:S center. Less than 0.2 residue of disulfide per mol of protein were found.

In the experiments described above, IAA was included simultaneously with the chelator and nucleotide. As noted, IAA increases the rate of center decomposition which could bias the labeling pattern of the cysteines. In complementary experiments, the apo-protein was generated without IAA and the stability of the apo-form was studied by carboxymethylation. Av2 was incubated with MgATP and α,α' -dipyridyl for 30 min prior to the addition of [^{14}C]IAA. As shown in Table IV, A, only residue 132 was substantially labeled and the total carboxymethylation of the protein was half that when IAA was included initially. The failure to label residue 97 suggests that apo-Av2 may have more than one conformation. For example, if apo-Av2 has a lower affinity for MgATP than does the holoenzyme, the predominant protein species after Fe center chelation may be the nucleotide-free structure, *viz.* a structure with residue 97 buried. To test this hypothesis, apo-Av2 was generated as above without IAA. After 30 min, the Mg and ATP concentrations were increased so that the solution contained Mg_2ATP , a tight binding inhibitor, and [^{14}C]IAA was added. As shown in Table IV, B, both residues 97 and 132 were labeled at a level similar to the earlier experiments where IAA was included with the chelator. Thus, apo-Av2, as well as holo-Av2, appears to undergo a nucleotide-dependent reversible conformational change.

Which Thiols in Av2 Are Exposed by Denaturing Solvents?—Although the Av2 Fe:S center is decomposed in solutions of

TABLE III

Carboxymethylation of Av2 by 9.5 mM [^{14}C]IAA in pH 8.0 Tris·HCl buffer containing 3.4 mM α,α' -dipyridyl

At four time points, the reaction was stopped and peptide maps generated. The fraction of labeling for each residue was calculated by equation 1. A, incubation mixture contained 3.8 mM MgATP; B, incubation mixture contained 3.8 mM MgADP.

Reaction time	Protein residue number							% Carboxy-methylation
	5	38	85	97	132	151	184	
<i>min</i>	<i>mol modified/mol protein</i>							
A. Av2 + MgATP (in α,α' -dipyridyl)								
1.0	0.06	0.05	0.03	0.14	0.17	0.03	0.05	7.6
2.5	0.07	0.07	0.07	0.32	0.30	0.10	0.07	14.3
10.0	0.15	0.14	0.18	0.61	0.71	0.17	0.13	29.9
30.0	0.17	0.21	0.37	0.80	0.89	0.19	0.18	40.1
B. Av2 + MgADP (in α,α' -dipyridyl)								
1.0	0.05	0.05	0.02	0.04	0.06	0.07	0.06	5.0
2.5	0.09	0.08	0.04	0.17	0.18	0.08	0.11	10.7
10.0	0.14	0.17	0.23	0.61	0.59	0.17	0.14	29.3
30.0	0.10	0.38	0.64	0.83	0.88	0.16	0.17	45.1

TABLE IV

Carboxymethylation of apo-Av2 in pH 8.0, 0.1 M Tris·HCl buffer containing 3.4 mM α,α' -dipyridyl

A, Av2 was incubated with 2.4 mM MgATP for 30 min before adding 9.5 mM [^{14}C]IAA for 30-min reaction; B, Av2 was incubated with 2.4 mM MgATP for 30 min after which the concentration of Mg and ATP are increased to the equivalent of 9.3 mM Mg_2ATP for 15 min. Carboxymethylation was for 15 min with 9.5 mM [^{14}C]IAA.

Condition	Protein residue number							% Carboxy-methylation
	5	38	85	97	132	151	184	
	<i>mol modified/mol subunit</i>							
A. apo-Av2 + 2.4 mM MgATP	0.11	0.12	0.20	0.23	0.48	0.11	0.13	19.7
B. apo-Av2 + 9.3 mM Mg ₂ ATP	0.12	0.11	0.09	0.63	0.72	0.13	0.13	27.6

TABLE V

Carboxymethylation of Av2 by 6.5 mM [^{14} C]IAA in 64% hexamethylphosphoramide, 0.1 M pH 8.0 Tris-HCl buffer
 A, [^{14} C]IAA was included with denaturant when the protein was added; B, Av2 was incubated in the denaturant for 20 min in the denaturant before adding [^{14} C]IAA.

Time after adding IAA min	Protein residue number						% Carboxymethylation
	5	38	85	97	132	151	
	mol modified/mol subunit						
A.							
5	1.08	0.30	0.40	0.46	0.30	0.39	55.6
30	1.03	0.49	0.58	0.77	0.43	0.45	66.9
B.							
5	0.47	0.47	0.42	0.43	0.20	0.45	41.6
30	0.74	0.72	0.68	0.63	0.60	0.77	70.4

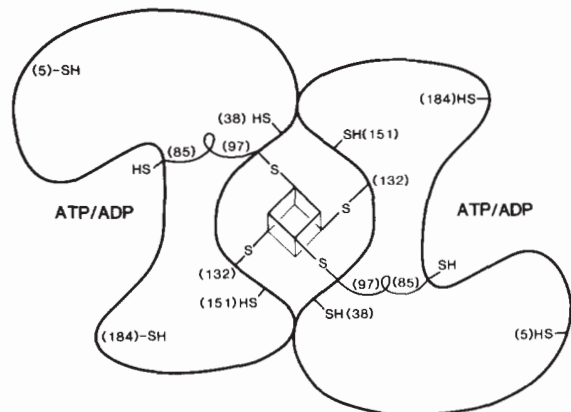


FIG. 4. Summary of thiol reactivity in Av2. The shape of subunits are drawn to indicate a 2-fold symmetry about the Fe:S center. Positions of the thiols are only to suggest relative reactivity at the Fe:S center and protection from IAA modification.

guanidine HCl (see above), the cluster can be extruded by thiol exchange in 60% hexamethylphosphoramide and is stable under these conditions (7). We have found that thiol ligands in many proteins, *e.g.* rubredoxin (23) and ferredoxins,² are only slowly alkylated when the protein is dissolved in denaturants. In an attempt to label nonligand thiols in Av2, the protein was dissolved in hexamethylphosphoramide and ~15% absorbance loss at 410 nm occurred during the first 2 min. If [^{14} C]IAA was included initially with denaturant, only residues 5 and 184 were quantitatively labeled (see Table V, A) and the equivalent of 94% of the nonligand thiols (67% of all cysteines) were alkylated in 30 min. Among the 5 partially labeled cysteinyl residues, the level of protection did not discriminate between the probable ligands and other residues. As shown in Table V, B, if the protein was denatured for 20 min before adding IAA, 42% of all cysteines were carboxymethylated in 5 min. This corresponds to 60% of the nonligand thiols with only residue 132 being partially protected. After 30 min, all cysteines were labeled at 0.60–0.79 mol per mol of Av2.

The striking feature of these labeling patterns is the absence of complete protection for any residue. It appears that Av2 has two classes of nonligand thiols. Residues 5 and 184 can be rapidly and quantitatively alkylated when the protein is denatured in the presence of IAA; yet they can exchange with center ligands in the absence of IAA. In contrast, residues 38, 85, and 151 rapidly exchange with center ligands before complete denaturation of the protein and are only partially labeled by IAA. The rapid exchange between these residues and the ligands indicates that other cysteines may be near the Fe:S center in the three-dimensional structure. However, the rapid exchange of ligands in the denatured protein precludes the

use of IAA under these conditions to selectively modify non-ligands and, thereby, to identify authentic ligands.

CONCLUSIONS

Our conclusions and speculations about the Av2 structure are summarized in Fig. 4.

1. Av2 contains no disulfides, hyperreactive thiols, or surface thiols as defined by reactivity with IAA.

2. Cysteinyl residues 97 and 132 are the probable thiol ligands for the Fe:S center in reduced Av2. The residues were selectively labeled in parallel to chromophore destruction in the native protein. Furthermore, they were the only residues modified during the MgATP-dependent chelation of the center. Because both residues were >80% modified, they account for 4 thiol ligands per mol of Av2. Our results are consistent with the proposal that a single 4Fe:4S center is shared between two subunits (7). We add that the center is symmetrically shared by the subunits, *viz.* the same cysteines in each subunit are ligands. Our results cannot exclude a model where the putative thiol ligands are chemically buried upon destruction of the Fe:S center while residues 97 and 132 are newly exposed.

3. Cysteinyl residue 85 appears to be associated with the nucleotide-binding site such that MgATP (or Mg₂ATP) protects the residue while MgADP protects to a lesser degree. It should be emphasized that residue 85 is not an exposed chemically reactive residue but reacted as the Fe:S center was destroyed. Our results do not distinguish between a thiol in the nucleotide site and a thiol that is allosterically protected. If cysteine 85 is in the nucleotide-binding site, it may be the nucleophile for ATP hydrolysis. Based upon secondary structure predictions, we (3) suggested that both residues 85 and 97 were unlikely to be ligands to the same Fe:S center. However, the predicted helical structure between these residues might bridge the Fe:S center and ATP site. The labeling experiments are consistent with the earlier proposal.

4. Our results support the hypothesis that a major conformational change occurs in Av2 upon binding MgATP. Furthermore, apo-Av2 undergoes a reversible conformational change induced by nucleotides. The labeling results with the apo-Av2 indicate that residue 97 is more susceptible to the conformational change and may be located deep in the Fe:S pocket while residue 132 is located toward the surface.

5. If the Av2 protein structure is denatured, cysteinyl residues 5 and 184 can be rapidly and quantitatively carboxymethylated while residues 38, 85, and 151 rapidly ligand-exchange with those of the Fe:S center (residues 97 and 132). This distinction between the two groups of residues suggests that residues 38, 85, and 151 may be near the Fe:S center in the three-dimensional structure, whereas residues 5 and 184 may be near the protein surface and distant from the Fe:S center.

6. Residues 38, 85, 97, 132, and 184 are common to all known Fe-protein sequences (see Table I). Two of these

residues in Av2 are probable Fe:S ligands in the reduced protein (residues 97 and 132) while another may be part of the nucleotide-binding site (residue 85). It is likely that these residues have the same function in all nitrogenase Fe-proteins. The other homologous cysteinyl residues might also have a specific function, which, at present, is undefined, *e.g.* electron transfer might involve ligand exchange with one of the other common cysteinyl residues.

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