Proc. Natl. Acad. Sci. USA
Vol. 77, No. 7, pp. 3826–3830, July 1980
Biochemistry

Comparison of the iron proteins from the nitrogen fixation complexes of *Azotobacter vinelandii, Clostridium pasteurianum,* and *Klebsiella pneumoniae*

(iron–sulfur proteins/cysteinyl peptides/nitrogenase)

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Communicated by John Buchanan, March 31, 1980

ABSTRACT **The molecular weights, amino acid compositions, amino- and carboxyl-terminal sequences, and ion-exchange peptide maps of the cysteine-containing tryptic peptides were determined for the iron proteins from the nitrogen fixation complexes of *Azotobacter vinelandii* (Av2) and *Klebsiella pneumoniae* (Kp2). Our results are compared to the known amino acid sequence of the iron protein from *Clostridium pasteurianum* (Cp2) [Tanaka, M., Haniu, M., Yasunobu, K. & Mortenson, L. E. (1977) *J. Biol. Chem.* 252, 7093–7100]. Previous studies have shown the iron proteins to have similar enzymatic functions and spectroscopic properties. Furthermore, the DNAs coding for the iron protein from many different species cross-hybridize [Ruvkun, G. B. & Ausubel, F. M. (1980) *Proc. Natl. Acad. Sci. USA* 77, 191–195]. Our results indicate that the protein structures are similar yet have significant differences. The amino-terminal sequences of Av2 and Kp2 are extended compared to the amino-terminal methionine of Cp2 and may indicate a different initiation site in these proteins. The amino-terminal sequences for Av2 and Kp2 are more homologous with each other than either of these are with Cp2. The carboxyl-terminal sequences are extended in Av2 (14 residues) and Kp2 (≈30 residues) compared to Cp2. The amino- and carboxyl-terminal sequences establish that either the structural gene sizes are different in the three organisms or extensive posttranslational modification must occur in some species. Because cysteinyl residues are involved at the active site of the iron protein, a sensitive peptide mapping technique was used to compare cysteinyl peptides of the iron protein from the three species. Av2 and Kp2 have a redistribution of cysteinyl residues when compared to Cp2. Three important differences in the cysteine distributions were found, namely, residue 4 is valine and residue 148 is alanine in Cp2, but cysteinyl residues occupy these positions in Av2, whereas residue 231 is cysteine in Cp2 but alanine in Av2. The peptide mapping technique provides a method for the investigation of selective chemical modification of cysteinyl residues.**

Biological reduction of dinitrogen to ammonia is catalyzed by a complex of two proteins: the molybdenum–iron (MoFe)-protein,† which contains the site of substrate reduction, and the iron (Fe)-protein, which reduces the MoFe-protein concomitant with ATP hydrolysis (1). The MoFe protein is an $\alpha_2\beta_2$ tetramer (M_r 220,000) and has 30–32 Fe atoms, 2 Mo atoms, and inorganic sulfur. The Fe-protein is a dimer (M_r 58,000–73,000) and has a single 4Fe–4S cluster, in which four cysteinyl residues between the subunits presumably function as ligands (1). The amino acid sequence for the Fe-protein from *C. pasteurianum* has been determined (2), whereas only limited sequence information is available for the MoFe-protein from *A. vinelandii* (3) or from *K. pneumoniae* (1).

Unlike other fundamental biological processes, nitrogen fixation is limited to a few, ecologically diverse, prokaryotes. The distribution of nitrogen-fixing organisms includes anaerobic, aerobic, and facultative anaerobic species which may have diverged as long as 2–3 billion years ago, a period when biological nitrogen fixation was not required because abiological ammonia was plentiful (4, 5). Yet, all species within a genus or family—*e.g., Klebsiella*—may not fix nitrogen. Many of the enzymatic and spectroscopic properties of the nitrogenase proteins from diverse species are similar, which suggests that the proteins are structurally similar (1). For example, Emerich and Burris (6) have shown that the Fe-protein from many species will form an active nitrogenase complex with the MoFe-protein from other species. These kinds of observations have led Postgate (7) to propose that nitrogen fixation may be a more recent evolutionary development for which the genes were distributed between species by plasmids. Support for this hypothesis was recently provided by Nuti *et al.* (8), who found that some of the nitrogen-fixation genes (*nif* genes) in *Rhizobium* are on plasmids. In addition, Ruvkun and Ausubel (9) have found by DNA hybridization that the DNA coding for part of the *nifD* gene [the α subunit in the MoFe-protein (10)] and for part of the *nifH* gene [the Fe-protein (10)] is highly conserved among 19 nitrogen-fixing species.

Although the nitrogenase complexes from various species appear to be similar, there is insufficient structural information about the proteins from the different organisms to make a direct comparison of the proteins. Indeed, in contrast to the observations cited above, there are some significant differences in the nitrogenase proteins from various species. For example, a notable exception to formation of active heterocomplements is the Fe-protein of *C. pasteurianum*; Cp2 can form only a 15%-active complex with Kp1 and forms an inactive heterocomplex with Av1 or most other MoFe-proteins. Furthermore, there could be a difference of as much as 5000 between the molecular weights of Cp2 and Kp2 (2, 11). There is a large variation in the reported number of cysteinyl residues per subunit for Kp2 (nine residues) and Av2 (two residues) (11, 12). There is a substantial difference in the temperature stability of the Fe-proteins from various sources (13). Finally, there appear to be differences among species in the biological regulation of the Fe-protein; e.g., in *Rhodospirillum rubrum* the Fe-protein, as isolated, must undergo activation by a peptide factor that requires ATP and divalent metal ion (14).

It is our purpose to describe some of the structural similarities

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† Nomenclature: MoFe-protein is equivalent to component 1; Fe-protein is equivalent to component 2. The abbreviations of these proteins, according to species, are: Av1, Cp1, and Kp1, Mofe-proteins from *Azotobacter vinelandii, Clostridium pasteurianum,* and *Klebsiella pneumoniae,* respectively; Av2, Cp2, and Kp2, Fe-proteins from *A. vinelandii, C. pasteurianum,* and from *K. pneumoniae,* respectively.

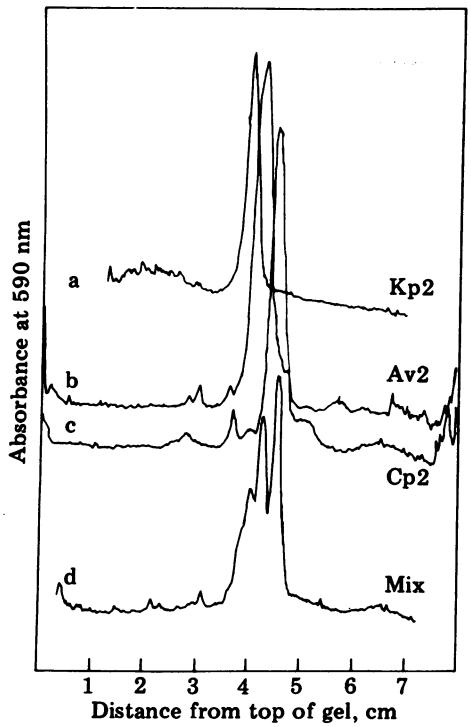

FIG. 1. Densitometer scans of gels from sodium dodecyl sulfate/polyacrylamide gel electrophoresis for the Fe-proteins on 7.5% gels under reducing conditions. Samples included: scan a, Kp2; scan b, Av2; scan c, Cp2; and scan d, a mixture of the three. Each protein sample (5–10 μg) was incubated in 8 M urea overnight at 37°C. The samples were heated at 37°C for 30 min in 1% sodium dodecyl sulfate/1% 2-mercaptoethanol before electrophoresis at pH 7.2. A GCA/McPherson spectrophotometer equipped with a linear transport scanner was used to scan at 590 nm.

and differences in the Fe-protein from three representative species: *A. vinelandii*, an aerobe, *K. pneumoniae*, a facultative anaerobe, and *C. pasteurianum*, an obligate anaerobe. Four points of comparison between these proteins were chosen: the amino-terminal sequences, the carboxyl-terminal sequences, the molecular weights, and the sequences of the cysteinyl regions in the protein. We report that the amino-terminal sequences of Av2 and Kp2 are extended one residue compared to the amino-terminal methionine in Cp2. In addition, the carboxyl terminals of Av2 and Kp2 are extended when compared to Cp2. These results indicate that the Fe-proteins of some or all species undergo posttranslational processing and that the gene size may be different in the various species. The results of gene DNA sequence determination should elucidate this question (cf. refs. 9 and 15). A second major distinction between the different Fe-proteins was found for the distribution of the cysteinyl residues. Because the cysteinyl residues are ligands for the 4Fe–4S centers in the protein, the changes in cysteinyl residue location may provide the basis for enzymatic differences.

MATERIALS AND METHODS

The Fe-protein from *C. pasteurianum* was the generous gift of W. Orme-Johnson. The protein from *K. pneumoniae* was provided by B. E. Smith and was further purified by ion-exchange chromatography on DEAE-cellulose, using pH 6.0, 0.1 M imidazole buffer that contained 8 M urea. The Fe-protein from *A. vinelandii* was isolated in our laboratory by the method of Kleiner and Chen (12). Sequence determination methods were those described previously (16). All other procedures are described in appropriate figure and table legends.

Table 1. Amino acid compositions of Fe-proteins

Amino acid	Residues per molecule				
	Cp2*	Kp2†	Kp2‡	Av2‡	Av2§
Aspartic acid	22	29	29	30	31
Threonine	13	17	18	12	13
Serine	13	10	12	10	10
Glutamic acid	35	41	38	35	43
Proline	9	9	9	9	9
Cysteine	6	8	9	7	2
Glycine	32	29	29	28	26
Alanine	20	30	29	28	27
Valine	19	21	23	25	25
Methionine	11	16	15	13	10
Isoleucine	20	22	23	20	19
Leucine	26	21	21	23	24
Tyrosine	12	8	9	9	7
Phenylalanine	5	6	7	7	7
Histidine	2	2	3	3	3
Lysine	16	16	16	16	11
Arginine	12	13	13	14	13
Tryptophan	0	0	0	0¶	0
Total	273	298	303	289	281

* From sequence determination by Tanaka *et al.* (2).
† Calculated from Eady *et al.* (11), using our value of M_r, 32,600.
‡ Average of duplicate hydrolysis of 24, 48, and 72 hr in 6 M HCl at 110°C. Molecular weights were taken as 32,600 for Kp2 and 31,200 for Av2. Serine and threonine were determined by extrapolation to initial time. Valine and isoleucine are the 72-hr values. Cysteine was determined as carboxymethylcysteine.
§ Calculated from Kleiner and Chen (12), using our value of M_r, 31,200.
¶ Tryptophan was determined after hydrolysis in 4 M methanesulfonic acid for 24 and 48 hr.

RESULTS AND DISCUSSION

In order to compare the sizes and the numbers of amino acid residues in the Fe-proteins, the molecular weights of Kp2 and Av2 were reinvestigated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The densitometer scans of gels for Kp2, Av2, and Cp2, and a mixture of all three proteins are shown in Fig. 1. Using the calculated molecular weight from the amino acid sequence of Cp2 (M_r 29,685)‡ and other marker proteins, a molecular weight of 31,200 was calculated for Av2 [cf. M_r 33,000 reported before (12)]. Amino acid analysis of Av2 based upon its larger molecular weight suggested 16 additional residues compared to Cp2 (Table 1). Kp2 was calculated to have a molecular weight of 32,600 [cf. M_r 34,600 reported before (11)] or ≈30 more residues than Cp2 (see Table 1). The amino acid compositions for all three proteins are compared in Table 1. Our values for Cp2 and Kp2 agree well with the previous reports (2, 11). However, our analysis of Av2 differs for three residues; we found 5 more lysyl residues, 8 fewer glutamyl residues, and 5 more cysteinyl residues than previously reported (12). Our analysis of cysteine was confirmed by the sequences of the cysteine-containing peptides given below.

DNA hybridization studies have suggested strong sequence homology for the Fe-protein from different species (9, 15). In order to determine the extent of homology, the sequences of the amino-terminal, carboxyl-terminal, and cysteine-containing internal regions were determined. The amino-terminal sequences for Av2 and Kp2 were determined by repetitive automated Edman degradation and are shown in Fig. 2. The sequences are homologous with Cp2 but have significant dif-

‡ The molecular weight from Cp2 was reported to be 28,657 from amino acid sequence analysis (2) and has been recalculated to be 29,685 (L. Mortenson, personal communication).

ferences. Av2 and Kp2 sequences are identical except for the amino terminal residue and are extended by one residue compared to Cp2. Because the amino-terminal sequence of Cp2 begins with methionine, one might expect that this methionine was the initiation site for the Fe-protein. Our finding that the Av2 and Kp2 proteins are extended by one residue and do not begin with methionine suggests that some or all Fe-proteins must undergo posttranslational modification. The processing may be either chain elongation or limited proteolysis of a precursor. Indeed, Cp2 may also be processed, and the finding of its amino terminal as methionine may be fortuitous. The second major difference between Cp2 and Av2 or Kp2 was the replacement of valine (residue 4 in Cp2) with cysteine. The finding of a single amino-terminal sequence for Av2 and for Kp2 confirmed that these two proteins, as for Cp2 (2), have identical subunits.

The carboxyl-terminal sequence for Av2 has been determined and is compared with that of Cp2 in Fig. 3. The sequence of a tryptic peptide from Av2 that is analogous to the carboxyl terminal of Cp2 has been determined (unpublished data); it extends 8 residues longer than the same peptide in Cp2. The extension could have resulted from a single base change between the chain-termination codon (UAG or UAA) and the resulting glutamic acid codon (GAG or GAA). The Av2 sequence is extended an additional 5 residues beyond the tryptic peptide. The total of 13 additional residues at the carboxyl terminal and the extra residue at the amino terminal account for 14 of the 16 extra residues predicted by amino acid analysis and molecular weight determination. That is: there should be few, if any, insertions or deletions in the internal sequence. We have confirmed this prediction by complete amino acid sequence analysis of Av2, which will be reported elsewhere. Although the peptides from the carboxyl terminal of Kp2 have not been isolated, the carboxyl-terminal sequence of Kp2 was deduced by carboxypeptidase A digestion (Fig. 3). The Kp2 carboxyl terminal is obviously different from the terminals of both Cp2 and Av2. This is consistent with the hypothesis that Kp2 and Av2 are extended at the carboxyl terminal compared to Cp2 and that Kp2 is longer than Av2. Because the carboxyl-terminal regions of Av2 and Kp2 are extended compared to Cp2, either Av2 and Kp2 are processed differently than Cp2 or the gene sizes for these three proteins are different. Furthermore, the extension at the carboxyl terminal will result in a different protein surface structure for Av2 or Kp2 than for Cp2. The change in structure might alter the subunit interactions or the Fe-protein–MoFe-protein interaction such that heterocomplements are inactive.

The electron transfer properties of the Fe-protein are due to the 4Fe–4S center, which is shared between the identical protein subunits (17, 18). The protein ligands for the cluster are cysteinyl residues and may represent a difference in the protein

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Av2   NH2-Ala-Met-Arg-Gln-Cys-Ala-Ile-Tyr-Gly-Lys-Gly-Gly-
Kp2   NH2-Thr-Met-Arg-Gln-Cys-Ala-Ile-Tyr-Gly-Lys-Gly-Gly-
Cp2   NH2-   -Met-Arg-Gln-Val-Ala-Ile-Tyr-Gly-Lys-Gly-Gly-

Av2       Ile-Gly-Lys-Ser-Thr-Thr-Thr-Gln-Asn-Leu-Val-Ala-Ala-
Kp2       Ile-Gly-Lys-Ser-Thr-Thr-Thr-Gln-Asn-Leu-Val-Ala-Ala-
Cp2       Ile-Gly-Lys-Ser-Thr-Thr-Thr-Gln-Asn-Leu-Thr-Ser-Gly-
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FIG. 2. The amino-terminal sequences of the Fe-proteins from *A. vinelandii*, *K. pneumoniae*, and *C. pasteurianum* (2). Sequences for carboxymethylated Av2 and Kp2 were determined by automated Edman degradation using the 0.1 M Quadrol program (16). For Av2 the initial yield was 40%, and the repetitive yield was 96%. For Kp2 the initial yield was 32%, and the repetitive yield was 98%.

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          262                                  273
Cp2   -Glu-Glu-Ile-Leu-Met-Gln-Tyr-Gly-Leu-Met-Asp-Leu-COOH

Av2   -Glu-Glu-Leu-Leu-Met-Glu-Phe-Gly-Ile-Met-Glu-Val-Glu-Asp-

Av2    Glu-Ser-Ile-Val-Gly-Lys-Thr-Ala-Glu-Glu-Val-COOH

Kp2                                    -( )-Phe-Ala-Ser-COOH
```

FIG. 3. Carboxyl-terminal sequences of Av2, Cp2, and Kp2. The carboxyl-terminal sequence of Fe-protein from *C. pasteurianum* is taken from Tanaka *et al.* (2). The carboxyl-terminal sequence of Fe-protein from *A. vinelandii* was ascertained from the sequence of tryptic peptides isolated by ion-exchange chromatography and gel filtration. The carboxyl-terminal sequence of Fe-protein from *K. pneumoniae* was determined from timed release of amino acids by carboxypeptidase digestion. The digestion used 5% enzyme (wt/wt) in 100 mM NH_4OAc, pH 9.0, at room temperature with removal of samples after 0.5, 1.5, and 3.0 hr. The samples were lyophilized, taken up in 50 mM citrate, and quantitated by amino acid analysis.

structure of the Fe-protein from various species. For the comparison of the cysteine regions in Av2 and Cp2, ion-exchange cochromatography was performed with the tryptic peptides from differentially radiolabeled carboxymethylated Fe-proteins. The differences between the Cp2 and Av2 cysteinyl peptides are readily seen in Fig. 4*A*. Namely, one peak of radioactivity equal to two cysteinyl residues was found in Cp2 but was missing in Av2, whereas three peaks of radioactivity equal to three cysteinyl residues were found in Av2 but not in Cp2. In order to identify which radiolabeled cysteinyl peptide corresponded to a specific sequence in the protein structure,

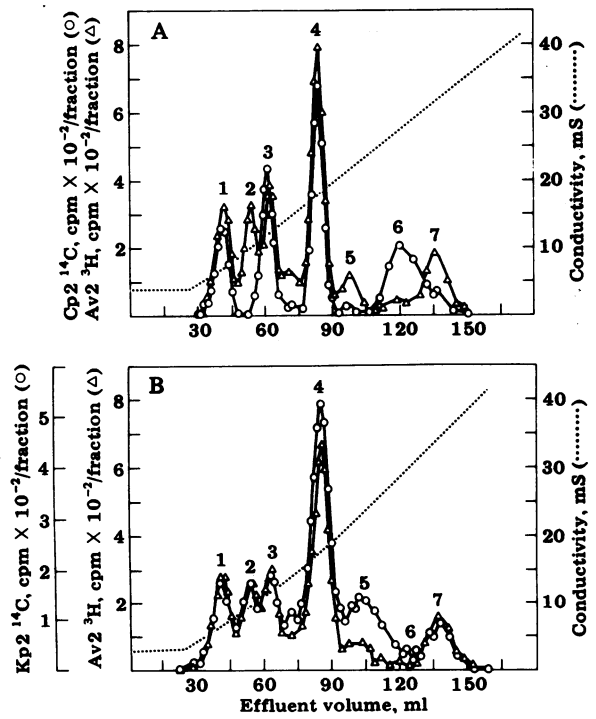

FIG. 4. Ion-exchange cochromatography of the tryptic peptides from Av2 and Cp2 (*A*) and from Av2 and Kp2 (*B*). Av2 was reduced and carboxymethylated with iodo[2-^{14}C]acetic acid (250 μCi/mmol; 1 Ci = 3.7 × 10^{10} becquerels) and Cp2 and Kp2 were separately reduced and carboxymethylated, each with iodo[2-^{3}H]acetic acid (500 μCi/mmol) (3). The mixtures of labeled proteins were digested with a total of 4% (wt/wt) trypsin over 4 hr in 0.1 M Tris·HCl at pH 8.0. The mixtures were each applied to a column of DEAE-Sephadex A-25 ion-exchange resin (1 × 20 cm) equilibrated in 0.1 M Tris·HCl, pH 8.0, and a linear 200-ml gradient was run to 0.55 M NaCl. More than 85% of each labeled cysteine was recovered.

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          3                   9
Cp2-A     Gln-Val-Ala-Ile-Tyr-Gly-Lys
Av2-A     Gln-Cys-Ala-Ile-Tyr-Gly-Lys   (Peak 2)

          32                      40
Cp2-B     Ile-Met-Val-Val-Gly-Cys-Asp-Pro-Lys
Av2-B     Val-Met-Ile-Val-Gly-Cys-Asp-Pro-Lys   (Peak 3)

          82                                                  97
Cp2-C     Cys-Val-Glu-Ser-Gly-Gly-Pro-Glu-Pro-Gly-Val-Gly-Cys-Ala-Gly-Arg
Av2-C     Cys-Val-Glu-Ser-Gly-Gly-Pro-Glu-Pro-Gly-Val-Gly-Cys-Ala-Gly-Arg   (Peak 4)

          126                                 137
Cp2-D     Asp-Val-Val-Cys-Gly-Gly-Phe-Ala-Met-Pro-Ile-Arg   (Peak 6)
Av2-D     Asp-Val-Val-Cys-Gly-Gly-Phe-Ala-Met-Pro-Ile-Arg   (Peak 7)

          144                       151
Cp2-E     Ile-Tyr-Ile-Val-Ala-Ser-Gly-Glu
Av2-E     Ile-Tyr-Ile-Val-Cys-Ser-Gly-Glu   (Peak 5)

          176                       184
Cp2-F     Leu-Gly-Gly-Ile-Ile-Cys-Asn-Ser-Arg
Av2-F     Leu-Gly-Gly-Leu-Ile-Cys-Asn-Ser-Arg   (Peak 1)

          227                             235
Cp2-G     Tyr-Asp-Pro-Thr-Cys-Glu-Gln-Ala-Glu-Glu   (Peak 6)
Av2-G     Tyr-Asp-Pro-Lys-Ala-Lys-Gln-Ala-Asp-Glu
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FIG. 5. Amino acid sequences of cysteinyl-containing regions of tryptic peptides from Cp2 and Av2. Regions are designated according to their order of occurrence from amino terminal to carboxyl terminal, with the numbering based upon Cp2 (2). Av2-G is a peptide derived from *Staphylococcus aureus* protease digestion of Av2 that does not contain cysteine but is homologous to the comparable region in Cp2. Peaks 1–7 refer to the tryptic peptides of Fig. 4 that contain these regions.

the radiolabeled peptides from Av2 were separated on a preparative-scale ion-exchange column and further purified by gel chromatography and paper electrophoresis (data not shown). The sequences of the cysteinyl regions from the Av2 tryptic peptides and their relative position in the Av2 sequence are shown in Fig. 5. Six of the seven cysteines were identified in tryptic peptides and corresponded to peaks 1–4 and 7 in Fig. 4. The seventh cysteine was in a tryptic peptide that eluted as a broad smeared peak centered at position 5.

Five of the Av2 cysteinyl residues exactly corresponded to five of the six cysteinyl residues in Cp2 (see Fig. 5). Namely, peptides F, B, and C have identical size and charge, and, as expected, the respective peptides from Av2 and Cp2 coeluted (peaks 1, 3, and 4, in Fig. 4*A*). In addition, the large, 40-residue, cysteine-containing, peptide D has three fewer carboxylic acid residues in Cp2 than in Av2 (data not shown); therefore, the two peptides have unique elution positions (peak 6 for Cp2 and peak 7 for Av2). However, three cysteinyl residue differences were found when comparing the Av2 and Cp2 peptides. Peptides Av2-A (peak 2) and Av2-E (broad peak 5) contained cysteinyl residues that were not present in the homologous regions of Cp2 (see Fig. 5). In contrast, region G in Cp2 had a cysteinyl residue that was not present in the comparable region of Av2. This peptide in Cp2 has only 1 less charge than peptide Cp2-D and is part of peak 6 in the Cp2 elution profile. The change of cysteine to alanine in Av2 was substantiated by the sequence of a peptide derived from *S. aureus* peptidase digestion of Av2 that overlapped the region. Although the sequences surrounding the cysteinyl replacements are highly conserved, all three alterations require two DNA nucleotide base changes at the cysteinyl codon; yet the identity in the cysteinyl regions (≈90%) is much higher than for the protein as a whole (≈60%).

A comparison map of the radiolabeled cysteinyl peptides from Kp2 and Av2 was prepared. As can be seen in the cochromatogram in Fig. 4*B*, Kp2 is indeed more nearly like Av2 than it is like Cp2. Seven of the nine cysteinyl residues in Kp2 are found in peptides that have the same size and charge as the seven cysteinyl residues from Av2. This includes peptides A–C and F as well as peptide D, which coeluted with the comparable peptide of Av2 (peak 7) rather than the peptide from Cp2 (peak 6). The similarity of the peptide maps extended to the broad peak 5. From the specific radioactivity in peaks 4 and 5 (68–115 ml, Fig. 4*B*) we calculate that there were 5.3 cysteinyl residues in this region for peptides from Kp2. Peak 4 contained the dicysteinyl peptide C, leaving three residues in the broad elution of peak 5. From our maps we could not determine whether the additional cysteinyl residues of Kp2 are in one peptide. However, the distribution of the radioactivity strongly suggested that Kp2 has a peptide similar to Av2-E and contains at least one cysteinyl residue.

The results presented here confirm that the Fe-proteins from different species have highly conserved amino acid sequences at the amino terminal and around the cysteinyl residues. For the three examples studied, five of the cysteinyl residues appear to be in identical sequences. However, the three differences involving cysteinyl residues found in Av2 appear to occur also in Kp2. Because Cp2 binds to the MoFe-proteins of *A. vinelandii* and *K. pneumoniae*, yet functions poorly or not at all in these heterocomplements, it is tempting to suggest that one or more of the cysteinyl residues that is different in Av2 and Kp2 may be involved in the reduction of the MoFe-protein. For example, Walker and Mortenson (19) have suggested that the 4Fe–4S cluster in the Fe-protein may undergo ligand exchange

during the MoFe-protein reduction cycle. Cysteinyl residues have also been implicated in the ATP-binding site (20). Thus, a cysteinyl difference in the initial cluster ligand, in a cluster exchange ligand, or in an ATP-binding site might account for the enzymatic difference in the Fe-proteins. The peptide mapping technique shown above should provide a method whereby specifically labeled cysteinyl residues—e.g., cluster residues or ATP-binding site residues—can be identified and compared between the different species.

The authors thank Dr. D. Lundell for many helpful discussions and for advice on the preparation of the Fe-protein from *A. vinelandii*. This work was supported by Grant 5901-0410-8-0106 from the Science and Education Administration of the U.S. Department of Agriculture. The purchase of the amino acid sequencer was, in part, supported by the Minnesota Medical Foundation and the University of Minnesota Graduate School Research Fund.

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