IRON-SULFUR CLUSTERS AND CYSTEINE DISTRIBUTION IN A FERREDOXIN FROM AZOTOBACTER VINELANDII

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SUMMARY In 80% dimethyl sulfoxide/H_2O, Azotobacter ferredoxin Fe-S clusters can be extruded with benzene thiol. The extruded clusters have an absorption spectra maximum at 458 nm which is characteristic of 4Fe-4S centers. The amino terminal sequence of the Azotobacter ferredoxin has 7 of the 8 Cys residues at residue numbers 8, 11, 16, 20, 24, 39 and 42. Except for Cys 24, all of these residues can be correlated to homologous Cys residues in other bacterial ferredoxins. Although two thirds of the first 45 residues are identical to or conservative replacements for the first 43 residues of other bacterial ferredoxins, the insertion of Cys-24 indicates a major change in the environment of one of the two 4Fe-4S clusters.

Iron-sulfur proteins have been demonstrated to participate as electron carriers in diverse biological reactions and have been classified by number of irons, redox potential, biological source, spectral properties and metabolic function (1). The most remarkable observation concerning these proteins has been the discovery that a 4Fe-4S high potential protein (HIPIP) from Chromatium (E_o=+350 mV) has a cubic iron-sulfur cluster very similar to the two 4Fe-4S clusters in bacterial ferredoxins (Fd) ($E_o \sim$ - 400mV) (2,3). Because of the substantial difference in the redox potential of the two proteins, Carter, et al. (4), proposed that 4Fe-4S clusters could have different formal oxidation states such that the clusters in oxidized Fd and reduced HIPIP should have a common intermediate oxidation state. In support of this postulate, Cammack (5) has shown that reduced HIPIP can be further reduced in dimethyl sulfoxide (DMSO) to a state with spectral properties similar to reduced Fd. Presumably, whether the cluster in the intermediate

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oxidation state becomes more oxidized (HIPIP) or more reduced (Fd) depends upon subtle differences in the environment of the cluster, e.g. the polarity of the protein side chains which interact with the cluster.

Recently, Sweeney, et al. (6) described the spectral and potentiometric properties of Fd I (iron-sulfur protein III (7)) of Azotobacter vinelandii. This protein, which is thought to participate in metabolic dinitrogen reduction, appears to have one 4Fe-4S cluster with $E_o \sim$ - 420mV and one 4Fe - 4S cluster with $E_o \sim$ + 340mV (6). That is: the protein has Fe-S clusters similar to both HIPIP and to Fd. Earlier work of Yoch and Arnon (8) indicated that this protein was considerably different from the other bacterial Fd's, viz., Azotobacter Fd has approximately twice the molecular weight of the other bacterial Fd's.

These reports have suggested to us several important questions:

(1) Is there chemical evidence to support 4Fe-4S clusters in Azotobacter Fd?

(2) Does the distribution of the cysteinyl residues in Azotobacter Fd resemble other bacterial Fd's, does it resemble HIPIP, or does it resemble both types of clusters? This may be the most important question concerning the Azotobacter Fd since it may be a prototype for large Fe-S enzymes such as nitrogenase which also have multiple 4Fe-4S centers with several different redox potentials and spectral properties (9).

(3) Is the amino acid sequence of Azotobacter Fd similar to other Fe-S proteins?

MATERIALS AND METHODS

Azotobacter vinelandii (Berkeley strain) ferredoxin I was isolated as described by Yoch and Arnon (8). Amino acid analysis, absorption spectra, molecular weight and biological properties were very similar to those already reported. Amino acid sequence analysis was performed by sequential Edman degradation using a Beckman 890c sequenator with modified dilute Quadrol buffer programs for peptides or for proteins (10). Tryptic and chymotryptic

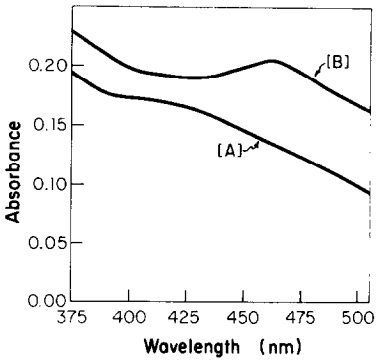

Figure 1. Absorption spectra of Azotobacter Fd I in 80% DMSO/0.1M, pH8.0 Tris HCl buffer (A) and 15 min after addition of ∿40 fold excess benzene thiol over protein Cys (B). Spectra did not change after first 5 min or upon the addition of more benzene thiol. Spectra were recorded in an anaerobic cell at room temperature using a Beckman 25 recording spectrophotometer.

peptides from reduced and carboxymethylated ferredoxin (RCM-Fd) were isolated by ion exchange chromatography on DEAE-Sephadex or gel chromatography on Sephadex G-25 (11). Amino acid residues were identified by two or three of the following methods; 1) as phenylthiohydantoin (PTH) derivatives using high pressure liquid chromatography (C_{18}upac from Water's Associates, T. Lorsbach and J. Howard, unpublished results), 2) as PTH-amino acids in the mass spectrometer, or 3) as the free amino acid after HI hydrolysis (11). All other methods are referenced in the text.

RESULTS AND DISCUSSION

Does Azotobacter ferredoxin have 4Fe-4S clusters?

Que, et al. (12) have shown that intact Fe-S clusters can be extruded from proteins under an anaerobic atmosphere in 80% DMSO/H_2O by displacing the protein cysteinyl residues with excess organic thiol. If benzene thiol is used as the ligand, then the absorption spectra of the cluster is shifted from ∿390 nm (Fd) to ∿458 nm (free 4Fe-4S clusters) or to ∿490 nm (free 2Fe-2S clusters) (12). Upon addition of benzene thiol to Azotobacter Fd in 80% DMSO, the absorption spectra (Fig. 1) shifted from a broad shoulder at 390-400 nm to a maximum at 458 nm. If one uses the ε_M (400nm) = 27,000 M^{-1} cm^{-1} for

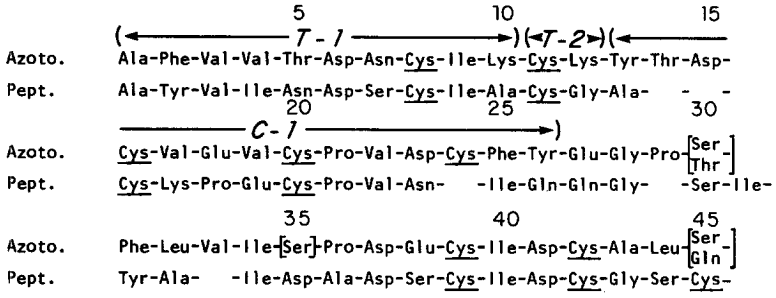

Figure 2. Amino acid sequence of Azotobacter Fd amino terminal region (Azoto.) and Peptococcus Fd (Pept.) (13). (T) are tryptic peptides and (C) are chymotryptic peptides. [ ] are residues which are not yet unambiguously established. Gaps have been inserted into the Peptococcus Fd sequence in order to maximize the homology of the two sequences.

Azotobacter Fd (8) and ε_M (458 nm) = 17,600 M^{-1} cm^{-1} for 4Fe-4S clusters (12), then one can estimate the release of 1.89 4Fe-4S clusters from the Azotobacter protein. This supports the postulate of Sweeney, et al. (6) that there are two 4Fe-4S clusters in the protein.

What is the distribution of cysteinyl residues in Azotobacter ferredoxin?

Figure 2 shows the amino terminal sequence which we have obtained for Azotobacter ferredoxin. The sequence was determined from three automated Edman degradation experiments using RCM-Fd (125 nmoles) or performate oxidized Fd (175 nmoles). For the whole protein (repetitive yield of 94-95%) 48 residues were determined with three ambiguous residues. Peptides from tryptic and chymotryptic digests of RCM-Fd have been isolated and three important peptides from the amino terminal region have been identified. These peptides were sequenced by sequential Edman degradation. The average repetitive yield for T-1 was 90% and for C-1 was 92% (based upon the first 12 residues of the peptide).

Figure 3 summarizes the distribution of cysteinyl residues and shows the Peptococcus ferredoxin sequence (13) for comparison. First, only Cys-8 and -11 are in an absolutely analogous position in the sequence. Second, the 3rd and 4th Cys residues in the sequence are probably analogous to the 3rd and 4th

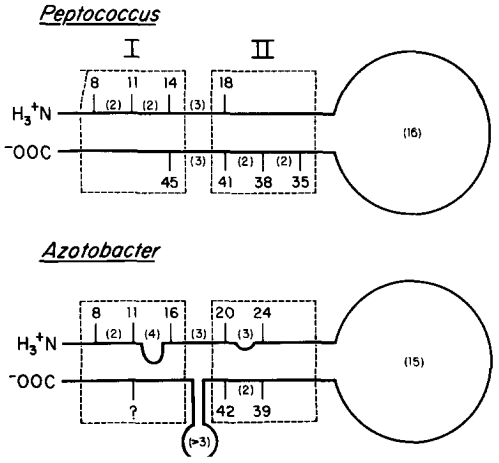

Figure 3. Distribution of cysteinyl residues in Azotobacter Fd and Peptococcus Fd (13). Numbers in ( ) are the number of residues between cysteinyl residues.

Cys residues in Peptococcus Fd. However, the spacing between the 2nd and 3rd Cys is substantially different, viz., four residues instead of two. Indeed, the two residues (-Gly-Ala-) between 2nd and 3rd Cys residues are invariant residues in all other 8Fe Fd's (14). Third, the most obvious change in the cysteinyl distribution is the 5th Cys residue. In all other 8Fe bacterial Fd's, the 5th Cys residue (residue 35) follows the large loop peptide (residues 19-34) and begins the second symmetrical group of Cys residues which are found in the second half of the protein molecule. In Azotobacter Fd, the 5th Cys is residue 24 and precedes the large loop peptide (residues 25 - 38 for Azotobacter). This is particularily striking since an invariant-Pro-Val-sequence (residues 19-20) follows the 4th Cys residue and is responsible for directing the peptide backbone into a loop (residues 19-34) behind and away from the Fe-S cluster in Peptococcus (2). Although this sequence is conserved in the Azotobacter Fd (residues 21-22), Cys-24 must alter the loop region. Finally, the 6th and 7th Cys residues in Azotobacter Fd are in an identical sequence to the 5th and 6th Cys residues of Peptococcus. We have not yet identified the exact location of the 8th Cys in our sequence but it is more than three residues removed.

We have traced our sequence over the known three-dimensional structure for Peptococcus Fd (2). From the substantial homology between certain parts of these sequences, we have concluded that for Azotobacter Fd, Cys residue numbers 8, 11, 16 and the last Cys are part of 4Fe-4S cluster I and residues 20, 24, 39 and 42 are part of cluster II. Although the grouping of Cys residues in Azotobacter Fd is analogous to other 8Fe Fd's, the symmetry and spacing of the Cys residues which are conserved for all other 8Fe Fd's is different in Azotobacter Fd (Chromatium Fd has a nonapeptide insertion at residues 40-50) (14). The substantial alteration in Cys distribution for cluster II in Azotobacter Fd may result in the formation of a HIPIP type cluster.

Does Azotobacter Fd have homology of sequence with other bacterial Fd's?

In addition to the homology of Cys residues 8, 11, 16, 20, 39 and 42 in Azotobacter Fd to comparable Cys residues in the bacterial Fd's, there are other regions of similarity. The most obvious of these similarities is the amino terminal 11 residues where 9 residues are identical with those found in one or more bacterial Fd's (14). Indeed, 25 of the 45 residues which we report are found in an analogous position in one or more bacterial Fd's (14). Most of these residues are invariant residues (14). There are an additional 3-5 residues in this region which could be considered conservative replacements. Many of these residues are found in the regions around the two Fe-S clusters of Peptococcus Fd (2), e.g., Phe-2, 31; Ile-9,34; and Val-4, 22 of the Azotobacter Fd sequence. Therefore, it seems reasonable to believe that the Azotobacter Fd is derived from the same ancestral Fd gene as the other 8-Fe bacterial Fd's. (14).

The sequence of the carboxyl terminal region of this molecule remains to be completed. The final 65 residues of the Azotobacter Fd may have arisen by gene duplication of the ∿50 amino acid residue ancestral gene (14); however, the carboxyl terminal region must be considerably different from the amino terminal region. For example, the amino terminal half contains 7 Cys and 2 basic residues whereas the carboxyl region contains 1 Cys and 7 basic residues.

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REFERENCES

1. Malkin, R., in Iron-Sulfur Proteins, W. Lovenberg, ed. (1973) Academic Press, N.Y., p. 1-26.
2. Adman, E., Sieker, L., and Jensen, L. (1973) J. Biol. Chem., 248, 3987-3996.
3. Carter, C., Kraut, J., Freer, S., Xuong, N-H., Alden, R., and Bartsch, R. (1974) J. Biol. Chem., 249, 4212-4225.
4. Carter, C., Kraut, J., Freer, S., Alden, R., Sieker, L., Adman, E., and Jensen, L. (1972) Proc. Nat. Acad. Sci., U.S.A., 69, 3526-3529.
5. Cammack, R. (1973) Biochem. Biophys. Res. Comm., 54, 548-554.
6. Sweeney, W., Yock, D., and Rabinowitz, J. (1975) J. Biol. Chem., 250, 7842-7847.
7. Shethna, Y. (1970) Biochem. Biophys. Acta, 205, 58-62.
8. Yock, D. and Arnon, D. (1972) J. Biol. Chem., 247, 4514-4520.
9. Münck, E., Rhodes, H., Orme-Johnson, W., Davis, L., Brill, W., and Shah, V. (1975) Biochem. Biophys. Acta, 400, 32-53.
10. Brauer, A., Margolies, M., and Haber, E. (1975) Biochem., 14, 3029-3035.
11. Howard, J., Fausch, M., and Nelsestuen, G. (1975) J. Biol. Chem., 250, 6178-6180.
12. Que, L., Holm, R., and Mortenson, L. (1975) J. Amer. Chem. Soc., 97, 463-464.
13. Tsundo, J., Yasunobo, K., and Whiteley, H. (1968) J. Biol. Chem., 243, 6262-6268.
14. Yasunobo, K., and Tanaka, M. in Iron-Sulfur Proteins, W. Lovenberg, ed. (1973) Academic Press, N.Y., p. 27-130.