The clostridial-type ferredoxins are iron-sulfur proteins (1-3) that function as electron carriers and have a molecular weight of approximately 6000 (4), contain eight atoms each of iron and acid-labile inorganic sulfur (5), and accept two electrons upon full reduction (6-11). X-ray crystallographic studies by Adman et al. (12, 13) have shown that the iron and inorganic sulfur in the clostridial-type ferredoxin from Peptococcus aerogenes occur in two "cube-like" iron-sulfur clusters approximately 12 Å apart. Each cluster consists of four iron and four inorganic sulfur atoms and is held to the peptide chain via cysteinyl sulfur atoms coordinated to each iron atom. The clusters are not in contact with water. The oxidation-reduction potentials of different clostridial-type ferredoxins have been reported to vary from -383 to -490 mv (8, 14), indicating that, even for proteins with the same cluster oxidation states, the peptide chain exerts an influence on the apparent oxidation-reduction potential. The single iron-sulfur cluster in Chromatium high potential iron-sulfur protein appears identical at the present x-ray resolution to the two (Fe,S,*)-clusters in P. aerogenes ferredoxin (15) yet the oxidation-reduction potentials of the two proteins are +350 and -427 mv, respectively (16, 17). The nature of the polypeptide chain must, therefore, have a profound effect on the oxidation-reduction properties of these (Fe,S,*)-containing proteins, since the geometry of the clusters is apparently the same. Carter et al. (15) have postulated that this difference in potentials arises from different formal oxidation states of the clusters in the two proteins. The peptide chain may, therefore, stabilize the particular cluster oxidation states normally present in each protein.

The amino acid sequences (18-26) of the clostridial-type ferredoxins show a high degree of homology. Generally the NH2-terminal residue of these proteins is alanine and aromatic amino acids (except for tryptophan) occur in two conserved positions in the peptide chain. The NH2-terminal residue of native ferredoxin from Clostridium acidi-urici ferredoxin is alanine (18), while the NH2-terminal residue of native ferredoxin from Clostridium pasteurianum is serine (19). The NH2-terminal residue of native ferredoxin from Clostridium acidi-urici ferredoxin is alanine (18), while the NH2-terminal residue of native ferredoxin from Clostridium pasteurianum is serine (19).
Solid citric acid (2 mmol) was then added. The cloudy mixture was treated with 2 ml (each time) of ethyl ether to remove excess reagents. The water was added and the reaction mixture was extracted four times with ethyl ether to facilitate the solubility of t-butylazidoformate. After 16 hours, 1 ml of t-butylazidoformate was dissolved in 2 ml of a 1:1 (v:v) solution of dioxane and water, the proportion of dioxane is increased to 1:5 (v:v) to ensure the formation of the Boc-dipeptide in solution. Dipeptides are usually more convenient to couple dipeptides, such as NH2-Ala'-Phe2, than C-terminal dipeptides. Before use, p-Dioxane, a product of Matheson, Coleman and Bell, was allowed to stand overnight in solution and was further purified by repeating the DEAE-cellulose column step. Most of the derivatives crystallized readily upon the addition of solid (NH4)2SO4 to 0.75 M solution of the derivatives. The C. acidi-urici apoferredoxin derivatives containing substitutions or deletions in the two NH2-terminal amino acid residues were reconstituted with iron and sulfide and purified by DEAE-cellulose chromatography as described by Hong and Rabinowitz (5) for native C. acidi-urici apoferredoxin. The more stable derivatives were well reconstituted and showed no loss in electron donation activity under anaerobic conditions. The yields of purified derivatives from the apoproteins were usually approximately 50% and 33%, respectively, for derivatives containing substitutions in positions 1 or 2.

Stability of Derivatives—The stability of the various derivatives was determined by measuring the rate of loss of activity in the phosphoroclastic assay system and the rate of loss of the derivatives A580 under conditions described below. Prior to the stability determination, the derivatives were repurified on a small DEAE-cellulose column. The C. acidi-urici apoferredoxin was dialyzed against 50 mM Tris-chloride buffer, pH 7.4, and adsorbed to the column. The column was washed with 50 mM Tris-chloride buffer, pH 7.4, and then with 0.50 M NaCl in 0.01 M Tris-chloride buffer, pH 7.4. The ferredoxin derivative was eluted with 0.45 M NaCl in 0.1 M Tris-chloride buffer, pH 7.4. This procedure removes traces of iron, sulfide, and apoprotein that might be present. The derivatives were diluted to a final concentration of approximately 10-8 M in a final buffer concentration of 0.1 M Tris-chloride, pH 7.4, containing 0.15 M NaCl. The solutions of the derivatives were then assayed several times with the aid of a mechanical vacuum pump and transferred anaerobically under argon to syringes that were stored at 4°C in a desiccator filled with argon. The first sample of each derivative removed from the syringe was used as the zero time sample for both the aerobic and anaerobic stability determination. This sample was used for the aerobic stability determination and was stored at 4°C in a test tube covered with paraffin. Subsequent samples for the "anaerobic" stability determination were removed each time from the syringes.

Nitrations of Feredoxin and Synthesis of [3-NH2-Tyr*]-Ferredoxin—The general nitration procedure described by Rioinan and Valsec (39) was used for the nitration of tyrosine in apoprotein. The Boc-dipeptide-N-hydroxysuccinimide ester was removed from the crystals of dicyclohexyl urea with a Pasteur pipet and was added dropwise to a room temperature solution of des-(Ala*-Tyr*). The mixture was then cooled in ice for at least 15 min. The solution of Boc-dipeptide-N-hydroxysuccinimide ester was removed from the crystals of dicyclohexyl urea with a Pasteur pipet and was added dropwise to a room temperature solution of des-(Ala*-Tyr*). The mixture was then cooled in ice for at least 15 min. The solution of Boc-dipeptide-N-hydroxysuccinimide ester was removed from the crystals of dicyclohexyl urea with a Pasteur pipet and was added dropwise to a room temperature solution of des-(Ala*-Tyr*). The mixture was then cooled in ice for at least 15 min. The solution of Boc-dipeptide-N-hydroxysuccinimide ester was removed from the crystals of dicyclohexyl urea with a Pasteur pipet and was added dropwise to a room temperature solution of des-(Ala*-Tyr*). The mixture was then cooled in ice for at least 15 min. The solution of Boc-dipeptide-N-hydroxysuccinimide ester was removed from the crystals of dicyclohexyl urea with a Pasteur pipet and was added dropwise to a room temperature solution of des-(Ala*-Tyr*). The mixture was then cooled in ice for at least 15 min. The solution of Boc-dipeptide-N-hydroxysuccinimide ester was removed from the crystals of dicyclohexyl urea with a Pasteur pipet and was added dropwise to a room temperature solution of des-(Ala*-Tyr*). The mixture was then cooled in ice for at least 15 min. The solution of Boc-dipeptide-N-hydroxysuccinimide ester was removed from the crystals of dicyclohexyl urea with a Pasteur pipet and was added dropwise to a room temperature solution of des-(Ala*-Tyr*). The mixture was then cooled in ice for at least 15 min.
des-(Ala'-Tyr')-apoferredoxin in order to obtain derivatives with Tyr** modified. Apoprotein (22 mg, 4 μmol) was dissolved in 20 ml of 0.05 M Tris-chloride buffer, pH 8.0. Then 50 μl (42 μmol, 5 times the excess over tyrosine) of 0.04 M tetranitromethane in ethanol (made by 1:10 dilution of tetranitromethane in 95% ethanol) was added and the solution stirred at room temperature for 2 hours. The yellow solution was then dialyzed overnight in the cold against a large volume of water with several changes of water. The nitrated apoprotein was stored as the lyophilized powder.

[3-NH-Tyr**]Ferredoxin was synthesized from des-(Ala'-Tyr')-apoferredoxin, by first nitrating Tyr** as described above and then adding the dipeptide Ala'-Tyr to des-(Ala'-Tyr'). [3-NO-Tyr**]apoferredoxin was then reduced to [3-NH-Tyr**]apoferredoxin by incubating the protein (5 mg/ml) in 8 M urea containing 0.07 M 2-mercaptoethanol, 0.004 M Na₂S, and a few grains of Na₂SO₄ for 2 hours prior to reconstitution. [3-NO-Tyr**]apoferredoxin treated in this way yielded a fairly stable derivative after reconstitution. However, [3-NH₂-Tyr**, Tyr']apoferredoxin, prepared by nitrating and reducing native apoferredoxin, did not yield a stable derivative upon reconstitution. [Leu',3-NH₂-Tyr']apoferredoxin was also synthesized, but did not yield a stable derivative upon reconstitution.

RESULTS

Spectra and Amino Acid Compositions—The various apoferredoxin derivatives were reconstituted and purified as described under "Methods." In Fig. 1 the spectrum of native C. acidi-urici ferredoxin is compared with the three general types of absorption spectra that were found for derivatives modified in position 1 or 2 of the peptide chain. Spectrum 2, Fig. 1 is that of [Lys']ferredoxin. It has the same characteristics as native ferredoxin shown in spectrum 3, Fig. 1. At the same concentration, the spectra of des-(Ala'-, [Ala'-], [Phe'-], [Lys'], [Leu'], [Met'], [Gly']-, and [Leu']mented ferredoxin appear indistinguishable from that of native C. acidi-urici ferredoxin. The ratio of A₄₃₈ to A₅₅₀ was approximately 0.78 to 0.79 for these derivatives and is 0.79 for this native ferredoxin. Spectrum 1, Fig. 1 is that of [Leu']ferredoxin and is similar to that of [Phe']-, [2-F-Phe']-, [3-F-Phe']-, and [His']ferredoxin. These derivatives contain Ala¹ and amino acids in position 2 that have a diminished absorbance at 280 nm compared to tyrosine. The A₄₃₈/A₅₅₀ ratio was approximately 0.81 to 0.82 for these derivatives. Spectrum 4, Fig. 1 is that of [Trp']ferredoxin. The A₄₃₈/A₅₅₀ ratio reflects the presence of Trp¹ instead of Tyr² and is 0.70 for this derivative. Spectrum 5, Fig. 1 is that of native C. acidi-urici apoferredoxin at the same concentration as holoferredoxin in spectrum 3, Fig. 1. As previously reported (5), most of the absorbance at 280 nm of ferredoxin is from the (Fe₄S₄)²⁺-clusters. However, as shown in Fig. 1 some information on the aromatic amino acid content of a purified ferredoxin may be derived from an examination of its absorption spectrum.

The amino acid compositions of the reconstituted derivatives with substitutions in positions 1 or 2 of the peptide chain are given in Tables I and II, respectively. Prior to analysis, the iron and sulfide were removed by precipitation of the protein with 5% trichloroacetic acid (5). The amino acid analyses of the apoferredoxin derivatives prior to reconstitution and purification were similar to those in Tables I and II. However, the yields of derivatives were generally lower than the 70% yield reported (5) for the reconstitution of untreated native C.acidi-urici apoferredoxin. This suggests that some degradative side reactions may occur during the synthesis. Material with apparently the correct amino acid composition, but that cannot be reconstituted to form a stable ferredoxin, may also be formed to a small extent. This material is removed by the purification procedure after reconstitution. These analyses and spectra indicate that the methods used are reliable for the preparation of these ferredoxin derivatives.

Activity of Derivatives in Phosphoroclastic Assay—The ability of the various derivatives to function as electron carriers in the phosphoroclastic assay system was determined. All of the derivatives containing substitutions or deletions in position 1 have the same activity as native C. acidi-urici ferredoxin (Fig. 2). The activity of derivatives containing substitutions in position 2 is shown in Figs. 3 and 4. [Trp']Ferredoxin has approximately 70% of the activity of native ferredoxin and was the only derivative including [3-NH₂-Tyr**, Tyr']apoferredoxin that exhibited activity significantly different from native ferredoxin in this assay system.

Activity of Derivatives in Cytochrome c Reduction Assay—In view of the suggestions that aromatic residues may function in electron transfer in clostridial-type ferredoxins (12, 28) and the possibility that electron transfer might not be the rate-limiting step in the phosphoroclastic assay system, the activity of some of the derivatives was tested in a ferredoxin-dependent cytochrome c reduction assay. This "artificial" system involves the reduction of ferredoxin by spinach ferredoxin-TPN reductase in the presence of TPNH. The reduction of cytochrome c by the reduced ferredoxin is measured by the increase in absorbance at 550 nm. In both the cytochrome c and phosphoroclastic assay systems ferredoxin acts catalytically. The activity of some of the derivatives tested in the cytochrome c assay is shown in Fig. 5. [Trp']Ferredoxin is also approximately 70% as active as native C. acidi-urici ferredoxin in this assay system, whereas [Phe'], [His'], [Gly'], and [Lys']ferredoxin are fully active. Preincubation experiments indicated that [Trp']Ferredoxin is stable for at least 10 min at the very dilute concentration used in this assay. Previously (30) [Leu']ferredoxin was shown to be essentially fully active in this assay system but is not as stable. Other derivatives tested in

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**Fig. 1.** The absorption spectra of the oxidized forms of C. acidi-urici ferredoxin are given in Table I and II, respectively. Prior to analysis, the iron and sulfide were removed by precipitation of the protein with 5% trichloroacetic acid (5). The amino acid analyses of the apoferredoxin derivatives prior to reconstitution and purification were similar to those in Tables I and II. However, the yields of derivatives were generally lower than the 70% yield reported (5) for the reconstitution of untreated native C. acidi-urici apoferredoxin. This suggests that some degradative side reactions may occur during the synthesis. Material with apparently the correct amino acid composition, but that cannot be reconstituted to form a stable ferredoxin, may also be formed to a small extent. This material is removed by the purification procedure after reconstitution. These analyses and spectra indicate that the methods used are reliable for the preparation of these ferredoxin derivatives.
Prior to analysis the iron and sulfide were removed by precipitating the protein with 5% trichloroacetic acid.

The diminution of the length of the polypeptide chain by a stabilities of derivatives with amino acid replacements in residue has a great affect on the stability of this ferredoxin. The acid after performic acid oxidation of native apoferredoxin. (21).

Synthesis of C. acidi-urici Ferredoxin Derivatives

Comparison of amino acid composition of native Clostridium acidi-urici ferredoxin and derivatives in position 1

Amino acid analysis was performed with a Beckman 117 automated analyzer after hydrolysis of the apoprotein for 22 hours in 6 N HCl at 110°. Prior to analysis the iron and sulfide were removed by precipitating the protein with 5% trichloroacetic acid.

| Amino acids | Native | des-Ala | [Ala'] | [Gly'] | [Pro'] | [Met'] | [Phe'] | [Leu'] | [Glu'] | [Lys'] |
|-------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Asp         | 7.88 (8) | 8.01   | 7.72   | 7.79   | 7.88   | 7.80   | 7.79   | 7.83   | 8.05   | 7.89   |
| Thr         | 0.95 (1) | 0.95   | 0.94   | 0.88   | 0.97   | 0.94   | 0.86   | 0.91   | 0.96   | 0.96   |
| Ser         | 2.73 (3) | 2.80   | 2.89   | 2.62   | 2.76   | 2.70   | 2.51   | 2.67   | 2.68   | 2.78   |
| Glu         | 4.06 (4) | 3.99   | 4.14   | 4.10   | 4.06   | 4.10   | 4.11   | 4.09   | 5.20   | 4.06   |
| Pro         | 4.00 (4) | 3.92   | 3.96   | 3.91   | 4.06   | 3.99   | 4.27   | 3.97   | 4.02   | 3.87   |
| Gly         | 3.91 (4) | 4.05   | 3.98   | 4.09   | 3.99   | 3.84   | 3.92   | 3.98   | 3.98   | 3.98   |
| Ala         | 8.70 (9) | 7.83   | 8.78   | 7.57   | 7.74   | 7.57   | 7.68   | 7.88   | 7.85   | 7.71   |
| Cysb        | 6.44 (8) | 4.94   | 6.74   | 5.98   | 6.35   | 6.64   | 5.70   | 6.29   | 6.31   | 6.38   |
| Val         | 5.00 (6) | 5.36   | 4.85   | 5.06   | 4.95   | 5.52   | 5.28   | 5.05   | 5.40   | 4.80   |
| Met         | <0.1 (0) | <0.1   | 1.05   | <0.1   | 1.02   | <0.1   | 1.63   | 1.87   | 1.15   |        |
| Ile         | 1.82 (2) | 2.00   | 1.95   | 1.82   | 1.75   | 1.90   | 1.90   | 1.66   | 1.47   |        |
| Leu         | <0.1 (0) | <0.1   | 1.05   | <0.1   | 1.05   | <0.1   |        |        |        |        |
| Lys         | <0.1 (0) | <0.1   | 1.05   | <0.1   | 1.05   | <0.1   |        |        |        |        |
| Arg         | 1.04 (1) | 0.97   | 0.95   | 0.99   | 1.05   | 1.02   | 1.00   | 1.00   | 0.86   | 1.04   |

The numbers in parentheses are the number of residues of each amino acid found in the sequence of C. acidi-urici ferredoxin by Rall et al. (21).

The cysteine values reported are those found in the normal hydrolysate and are generally approximately 75% of the value found for cysteic acid after performic acid oxidation of native apoferrerdoxin.

Stability of Derivatives—The stability of the various derivatives in solution was determined as described under "Methods." The decrease in activity with time of the derivatives was also examined, but it was a reliable indication of the integrity of the ferredoxin only until one-third to one-half of the initial A280 was lost. Thereafter, as previously reported by Hong and Rabinowitz (37) the biological activity of ferredoxin is lost more rapidly than the A280 indicating that degraded ferredoxin still has some residual A280. All of the derivatives except for [Ala']- and des-Ala'-ferredoxin are as active as native ferredoxin. Preincubation experiments indicated that in very dilute solutions, other experiments shown later also indicate that des-Ala' and [Glu']-ferredoxin are very unstable.

DISCUSSION

At least three types of iron-sulfur proteins that contain (Fe,S4)2+—clusters are known. Ferredoxins from Desulfovibrio gigas (40), Bacillus polymyxa (41, 42), and Desulfovibrio desulfuricans (43) most likely contain a single (Fe,S4)2+—cluster and have oxidation-reduction potentials of approximately -400 mv. The high potential iron-sulfur protein from Chromatium also contains a single (Fe,S4)2+—cluster (44) but has an oxidation-reduction potential of +350 mv (16). Chromatid-type ferredoxins, as shown for P. aerogenes ferredoxin (12, 13), contain two (Fe,S4)2+—clusters and have oxidation-reduction potentials in the range of -383 to -490 mv (8, 14). X-ray crystallographic analysis (15) indicates that the (Fe,S4)2+—cluster in Chromatium high potential iron-sulfur protein is structurally the same as the two (Fe,S4)2+—clusters in P. aerogenes ferredoxin despite the greatly different oxidation-reduction potentials of the two proteins. Herskovitz et al. (45) ...
TABLE II
Amino acid composition of Clostridium acidi-urici ferredoxin derivatives in position 2 and or 30 or both

Amino acid analysis was performed with a Beckman 117 automated analyzer after hydrolysis of the apoprotein for 22 hours in 6 N HCl at 110°. Prior to analysis the iron and sulfide were removed by precipitating the protein with 5% trichloroacetic acid.

| Amino acid | Ferredoxin | Native des- | P | Ala' | [His']- | [Trp']- | [Phe']- | [2-F-] | [3-F-] | NH3-Tyr* |
|------------|------------|-------------|---|------|---------|--------|--------|--------|--------|----------|
| Asp 8      | 7.94       | 7.93        | 7.89 | 7.83 | 7.90    | 7.86   | 7.89   | 7.85   | 7.89   | 7.97     |
| Thr 1      | 0.97       | 0.91        | 0.90 | 0.94 | 0.94    | 0.91   | 0.91   | 0.94   | 0.91   | 0.94     |
| Ser 3      | 2.58       | 2.82        | 2.80 | 2.86 | 2.68    | 2.75   | 2.84   | 2.75   | 2.84   | 2.84     |
| Glu 4      | 4.03       | 4.04        | 4.06 | 4.08 | 4.05    | 4.00   | 4.02   | 4.00   | 4.02   | 4.02     |
| Pro 4      | 4.08       | 3.99        | 4.02 | 3.93 | 3.97    | 4.03   | 4.00   | 4.00   | 4.00   | 4.00     |
| Gly 4      | 4.00       | 3.91        | 3.98 | 4.06 | 4.02    | 3.85   | 3.87   | 3.85   | 3.87   | 3.87     |
| Ala 8      | 7.92       | 6.09        | 6.01 | 5.75 | 8.71    | 8.06   | 8.51   | 8.51   | 8.51   | 8.51     |
| Cys 8      | 6.76       | 6.37        | 6.00 | 6.63 | 6.65    | 6.00   | 4.95   | 4.95   | 4.95   | 4.95     |
| Val 6      | 5.13       | 4.73        | 4.91 | 4.80 | 4.87    | 5.13   | 4.83   | 4.83   | 4.83   | 4.83     |
| Met 0      | 0          | 0           | 0   | 0    | 0       | 0      | 0      | 0      | 0      | 0        |
| Ile 5      | 4.44       | 4.06        | 4.13 | 4.18 | 4.65    | 4.17   | 4.35   | 4.35   | 4.35   | 4.35     |
| Leu 0      | 0          | 0           | 0   | 0    | 0       | 0      | 0      | 0      | 0      | 0        |
| Tyr 2      | 0.96       | 0.82        | 0.98 | 1.00 | 0.99    | 0.85   | 0.62   | 0.62   | 0.62   | 0.62     |
| Phe 0      | 0          | 0           | 0   | 0    | 0       | 0      | 0      | 0      | 0      | 0        |
| Lys 0      | 0          | 0           | 0   | 0    | 0       | 0      | 0      | 0      | 0      | 0        |
| His 0      | 0          | 0.88        | 0   | 0    | 0       | 0      | 0      | 0      | 0      | 0        |
| Arg 1      | 0.95       | 0.98        | 0.97 | 0.94 | 0.95    | 0.90   | 0.94   | 0.94   | 0.94   | 0.94     |

*The sequence of C. acidi-urici ferredoxin was determined by Rall et al. (21).
*With our analyzer, 3-F-Phe elutes in the same position as phenylalanine; therefore, there was no difficulty in quantitating the 3-F-Phe present in [3-F-Phe*]ferredoxin. However, 2-F-Phe elutes in the same position as tyrosine. The value reported for 2-F-Phe and Tyr for [2-F-Phe*]ferredoxin is one-half the value found that eluted in the position of tyrosine.
*Cysteine values reported are those found in the normal hydrolysate and are generally approximately 75% of the value found for cysteic after performic acid oxidation of native apoferredoxin.
*This value represents 3-NH3-tyrosine, which elutes near histidine and was clearly resolved from other amino acids.

proposed that the oxidation states of the (Fe,S,*)-clusters in the reduced form of high potential iron-sulfur protein and the oxidized form of P. aerogenes ferredoxin are the same. Carter et al. (15) have advanced a "three-state hypothesis" for the (Fe,S,*)-cluster and proposed that his difference in oxidation-reduction potentials of the two proteins may be explained by the existence of different sets of oxidation states of the (Fe,S,*)-clusters in each protein. Cammack (46) has provided support for this by showing that Chromatium high potential iron-sulfur protein in 70% aqueous dimethyl sulfoxide can be reduced with dithionite to a form that exhibits a reduced ferredoxin-like electron paramagnetic resonance signal. This oxidation state of high potential iron-sulfur protein is different from the reduced or oxidized forms isolated normally (16).

Holm and his colleagues (45, 47-50) have reported the synthesis and properties of model compounds containing the (Fe,S,*)-cluster. Of course, the synthesis of these compounds is proof that a peptide chain is not necessary for the existence of an (Fe,S,*)-cluster. However, to date, no water-soluble model compounds have been reported. Furthermore, it is not known how the peptide chain constrains the oxidation states normally present in proteins containing (Fe,S,*)-clusters and if there are important interactions of amino acid side chain groups with the (Fe,S,*)-cluster.

Generally, alanine has been found to be the NH3-terminal residue of clostridial-type ferredoxins (18-25). Peptostreptococcus elsdenii ferredoxin contains Met' and, to date, is the only known exception (26). In the structure of P. aerogenes...
ferredoxin determined by x-ray methods by Adman et al. (12), the $\alpha$-amino group of Ala$^1$ is hydrogen-bonded to the $\gamma$-carboxyl of Asp$^{37}$. The position of Asp$^{37}$ in P. aerogenes ferredoxin is homologous to position 39 of other clostridial-type ferredoxins and either Asp (18-21, 23, 24, 26) or Glu (22, 25) occur in this position in the peptide chain. The methyl group of Ala$^1$ in P. aerogenes ferredoxin is located between Asp$^{37}$ and Asp$^{44}$, the COOH terminus of the protein (12). The studies reported in this paper show that the presence or nature of the NH$_2$-terminal amino acid residue of C. acidi-urici ferredoxin does not affect the electron transfer ability of this protein in the assays tested (Figs. 2 and 4). The aerobic stability in aqueous solution of C. acidi-urici ferredoxin is, however, greatly affected by the nature of the NH$_2$ terminus of the protein (Table III). 

TABLE III

| C. acidi-urici ferredoxin | Aerobic | Anaerobic |
|--------------------------|---------|-----------|
|                           | $t_H$ (days) | % relative to native aerobic |
| Native                    | 54 100  | 185 342   |
| [Ala$^1$]-                | 56 104  | 162 300   |
| [Phe$^1$]-                | 50 93   | 111 206   |
| [Lys$^1$]-                | 47 87   | 140 259   |
| [Pro$^1$]-                | 47 87   | 97 160    |
| [Leu$^1$]-                | 47 87   | 74 137    |
| [Met$^1$]-                | 31 57   | 60 111    |
| [Gly$^1$]-                | 21 39   | 34 63     |
| [Glu$^1$]-                | 9 17    | 23 40     |
| des-Ala$^1$               | 1 2     | 3 6       |

* N.D., not detected.

The stabilities of C. acidi-urici ferredoxin and derivatives with amino acid replacements in position 1

The stabilities were determined as described in the text by following the loss in biological activity of ferredoxin and its derivatives. $t_H$ is equal to the time in days for one-half of the activity to be lost. The proteins were approximately $10^{-4} \text{ M}$ in 0.1 M Tris-chloride buffer, pH 7.4, containing 0.15 M NaCl.

FIG. 4. The activity in the phosphoroclastic assay of Clostridium acidi-urici native ferredoxin (●), [2-F-Phe$^1$]- (□), [3-F-Phe$^1$]- (△), and $\beta$-NH$_2$-Tyr$^*$-ferredoxin (×).

FIG. 5. The activity in the cytochrome c reduction assay of Clostridium acidi-urici native ferredoxin (●), [Gly$^1$]- (▽), [Lys$^1$]- (■), [His$^1$]- (×), [Phe$^1$]- (○), and [Trp$^1$]-ferredoxin (□).

Stability of Clostridium acidi-urici ferredoxin and derivatives with amino acid replacements in position 2

The stabilities were determined as described in the text by following the loss in biological activity of ferredoxin and its derivatives. $t_H$ is equal to the time in days for one-half of the activity to be lost. The proteins were approximately $10^{-4} \text{ M}$ in 0.1 M Tris-chloride buffer, pH 7.4, containing 0.15 M NaCl.

TABLE IV

| C. acidi-urici ferredoxin | Aerobic | Anaerobic |
|--------------------------|---------|-----------|
|                           | $t_H$ (days) | % relative to native aerobic |
| Native                    | 54 100  | 185 342   |
| [Trp$^1$]-                | 26 48   | 74 137    |
| [Phe$^1$]-                | 24 44   | 62 115    |
| [His$^1$]-                | 22 11   | 28 52     |
| [Leu$^1$]-                | 8 15    | 13 24     |
| [Pro$^1$]-                | 0.02 0.04 | |
| [Gly$^1$]-                | N.D.*   |           |
| des(Ala$^1$)-Tyr$^*$-      | N.D.    |           |

Stability of Clostridium acidi-urici ferredoxin and derivatives with amino acid replacements in position 2

The stabilities were determined as described in the text by following the loss in biological activity of ferredoxin and its derivatives. $t_H$ is equal to the time in days for one-half of the activity to be lost. The proteins were approximately $10^{-4} \text{ M}$ in 0.1 M Tris-chloride buffer, pH 7.4, containing 0.15 M NaCl.

| C. acidi-urici ferredoxin | Aerobic | Anaerobic |
|--------------------------|---------|-----------|
|                           | $t_H$ (days) | % relative to native aerobic |
| Native                    | 54 100  | 185 342   |
| [Trp$^1$]-                | 26 48   | 74 137    |
| [Phe$^1$]-                | 24 44   | 62 115    |
| [His$^1$]-                | 22 11   | 28 52     |
| [Leu$^1$]-                | 8 15    | 13 24     |
| [Pro$^1$]-                | 0.02 0.04 | |
| [Gly$^1$]-                | N.D.*   |           |
| des(Ala$^1$)-Tyr$^*$-      | N.D.    |           |
derivatives reported in this paper under anaerobic conditions increased their stability about 2- to 5-fold (Tables III and IV). This indicates that oxygen contributes to the destruction of ferredoxin.

All clostridial-type ferredoxins of known sequence contain either tyrosine (18, 20, 21), phenylalanine (19), or histidine (23, 24, 26) in position 2 of the peptide chain except for ferredoxins from the photosynthetic bacteria Chromatium (22) and Chlorobium limicola (25). These latter two ferredoxins contain Leu² and Tyr². However, they both contain more than the “normal” 55 amino acid residues present in most clostridial-type ferredoxins and at least two other aromatic residues occur in the peptide chain of each of these ferredoxins. We previously described the semisynthetic synthesis of a modified C. acidi-urici ferredoxin (30) and recently that of a modified Clostridium M-E ferredoxin (31). These two modified ferredoxins contain Leu² that has been substituted for a tyrosyl residue occurring in this position of the peptide chain of each native protein. Both of these [Leu²]ferredoxins are as active as an electron carrier as is native ferredoxin in the enzymatic assays tested. Since Clostridium M-E [Leu²]ferredoxin does not contain an aromatic residue, this shows that aromatic residues are not essential for electron transfer in clostridial-type ferredoxins. Therefore, as suggested earlier (30), the possibility should be considered that electron transfer occurs via cysteinyl sulfur atoms, some of which appear to be exposed to the solvent in P. aerogenes ferredoxin (12).

It was also found that neither C. acidi-urici des-(Ala¹-Tyr²) apoferredoxin, which lacks the two NH₂-terminal residues, or [Gly³]apoferredoxin, which contains Ala¹ and Gly³, form stable derivatives upon reconstitution (30). Since the x-ray (12) and ¹C-NMR (27-29) studies on clostridial-type ferredoxins show that the aromatic residue in position 2 of the peptide chain is near an (Fe₅S₅⁺)-cluster, the inability of these two derivatives to form stable derivatives indicates that the residue in position 2 acts as a hydrophobic shield for the cluster. Therefore, the peptide chain serves not only to solubilize the (Fe₅S₅⁺)-cluster in an aqueous solution but also to protect it from harmful species such as oxygen and probably water. This agrees with the observation of Malkin and Rabinowitz (51) that C. acidi-urici ferredoxin is more labile in aerobic than anaerobic solutions containing urea or guanidine hydrochloride. McDonald et al. (52) have also reported that C. pasteurianum ferredoxin is more oxygen-sensitive when the protein is perturbed with aqueous dimethyl sulfoxide. The proposal that one of the roles of the peptide chain is to shield the (Fe₅S₅⁺)-cluster is supported by recent experiments by Maskiewicz et al. (53, 54) that demonstrate that the (Fe₅S₅⁺)-cluster can hydrolyze via a mechanism involving attack by protons.

The data in this paper show that [Phe³]- and [His³]ferredoxins are as active as electron carriers as native C. acidi-urici ferredoxin in the two enzymatic assays tested (Figs. 3 and 5). In addition [2-NH₂-Tyr⁴], [2-F-Phe³], and [β-F-Phe³]ferredoxins are also fully active in the phosphorylascassic assay, which was the only assay in which they were tested. These results are in accord with our earlier evidence that aromatic residues are not essential for electron transfer in clostridial-type ferredoxins (30, 31), since some differences in rates might be expected for the derivatives reported in this paper if electron transfer via an aromatic residue were the rate-limiting step. [Phe³]ferredoxin is approximately one-half as stable as native C. acidi-urici ferredoxin (Table IV). Since [Phe³]ferredoxin differs from native ferredoxin only by the lack of a hydroxyl group on the aromatic ring of the residue in position 2, this decreased stability is somewhat surprising. Possibly, Phe³ does not shield the neighboring (Fe₅S₅⁺)-cluster as well as Tyr³, since the water structures around the residue in position 2 of the two proteins probably differ. Magnetic resonance data (27) suggest that the protein structure of [Phe³]ferredoxin is the same as native C. acidi-urici ferredoxin. It was suggested (55, 56) that the heat stability of ferredoxins from the thermophilic bacteria Clostridium tartaricurum and Clostridium thermosaccharolyticum might partially result from the presence of histidine in these ferredoxins and that the substitution of His³ for Tyr³ may stabilize clostridial-type ferredoxins, since His³ might be in a position to hydrogen bond to the sulfur atom of Cys⁴ (56). This hypothesis is not supported by the results reported here in which C. acidi-urici [His³]ferredoxin is only approximately 41% as stable as native ferredoxin.

Tryptophan has not been found yet in any native clostridial-type ferredoxin of known sequence. C. acidi-urici [Trp³]ferredoxin is the only ferredoxin derivative that is less active than native ferredoxin. It has approximately 70% of native ferredoxin activity in both the phosphorolytic and cytochrome c reduction assays. Although the rate-limiting step is not known in either of these relatively complex assays, this suggests that electron transfer and not binding is the rate-limiting step in these two assays, since different binding constants between the various enzymes in the two assay systems and [Trp³]ferredoxin might be expected. [Trp³]ferredoxin is possibly less active than native ferredoxin because the substitution of the larger tryptophan residue for Tyr³ has altered the conformation of this ferredoxin. The suggestion that the conformation of [Trp³]ferredoxin is altered is in accord with the decreased stability (48%) of [Trp³]ferredoxin and, as reported elsewhere (32), the different oxidation-reduction potential of [Trp³]ferredoxin relative to native C. acidi-urici ferredoxin.

The studies described in this paper show that the replacement of amino acid residues in the NH₂-terminal portion of C. acidi-urici ferredoxin has very little effect on the electron transfer ability of the protein in two enzymatic assays tested except in the case of [Trp³]ferredoxin. However, most of the substitutions decrease the stability of the protein in aqueous solution. It is likely, at least in some cases, that the decreased stability results from unfavorable interactions of amino acid side chain residues or exposure of the neighboring (Fe₅S₅⁺)-cluster to attack by harmful species such as oxygen and probably water. It is also possible, especially in the case of [Trp³]ferredoxin, that the gross conformation of some region of the protein may be altered and that this alteration decreases the stability of ferredoxin. As discussed in the following paper (32), alterations in the conformation of C. acidi-urici ferredoxin also may be responsible for the changes in oxidation-reduction potential of this protein depending on the particular amino acid replacement or ionic composition of the solution. Therefore, the peptide chain not only serves to solubilize the (Fe₅S₅⁺)-clusters and to protect them, but it also exerts a fine control on the oxidation-reduction potential of C. acidi-urici ferredoxin (32) and, as shown for [Trp³]ferredoxin in this paper, can also influence the biological activity of ferredoxin.

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