The Effects of Chemical Modifications on the Reconstitution, Activity, and Stability of Clostridial Ferredoxin*

(Received for publication, February 19, 1970)

J.-S. HONG† AND JESSE C. RABINOWITZ

From the Department of Biochemistry, University of California, Berkeley, California 94720

SUMMARY

Native ferredoxin isolated from Clostridium acidi-urici cannot be acetylated and is not digested by carboxypeptidase A. However, apoferredoxin, free of iron and sulfide, can be acetylated and the COOH-terminal alamine and glutamine residues are quantitatively removed by carboxypeptidase A. Ferredoxin derivatives can be reconstituted from both modified proteins. The following derivatives were also prepared: N-Acetimido-, N-succinyl-, tetraido-, various N-aminoacyl derivatives (glycyl-, phenylalaninyl-, lysyl-, glutamyl-, and methionyl-) and N-t-butyloxycarbonyl-. All modified apoferredoxins, except the N-succinyl- and the tetraido-derivatives, could be converted to the corresponding ferredoxin derivatives. These had both lower biological activity and stability than the native protein. Those with a positive charge at the NH₂-terminal end were more stable than those with no charge (acetyl-, t-butyloxycarbonyl-). Steric effects of the added amino acid derivatives were also detected. It is concluded that both the NH₂-terminal and the COOH-terminal amino acids are important for conferring stability to the ferredoxin structure.

EXPERIMENTAL PROCEDURE

Materials

The sources of the chemicals used were as follows: uniformly labeled ¹⁴C-acetic anhydride, New England Nuclear; ³²P, Nuclear-Chicago; urea, Baker Chemicals, purified as described by Margin and Merrifield (8); crystalline carboxypeptidase A (treated with difluorophosphate), Worthington; ethyl acetimido-hydrochloride and succinyl anhydride, Eastman; N-BOC-glycine-p-nitrophenyl ester, N-BOC-L-methionine-p-nitrophenyl ester, N-BOC-L-phenylalanine-p-nitrophenyl ester, N-BOC-L-glutamic acid-γ-benzyl ester, α-p-nitrophenyl ester, N,N-bis-BOC-L-lysine-p-nitrophenyl ester, Cyclic Chemicals; BOC-oxide and redistilled dimethyl formamide were generously supplied by

* This work was supported in part by Research Grant A-2109 from the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service.

† Part of the data was taken from a thesis submitted by J.-S. Hong to the University of California in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
Dr. D. Levy, Mr. S. M. Shigii, and Dr. F. H. Carpenter. Other chemicals were obtained from sources previously noted.

*C. acidii* urici was grown as previously described (9) except that sodium carbonate and sulfuric acid were omitted from the media. Previously described procedures were used for the preparation of ferredoxin (4), apoferredoxin (5), and apoferredoxin (5) from this organism, as well as for the assay of ferredoxin in an enzymatic test (10).

**Methods**

**Acetylation**—The procedure used was based on the method described by Fraenkel-Conrat (11). 14C-Acetic anhydride (uniformly labeled) (28 μl) was added in small increments over a period of 1 hour to apoferredoxin or native ferredoxin (18 mg) in 4 ml of 50% saturated sodium acetate at 0°, and the reaction was allowed to continue for another 1.5 hours. With the apoferredoxin, the mixture was then dialyzed for 30 hours at 4° with three changes of 0.01 M Tris-chloride buffer, pH 8.5 (3 liters each). In the case of the ferredoxin sample, the mixture was passed over a Sephadex G-25 column equilibrated with 0.1 M Tris-chloride buffer, pH 7.4, and the column was developed with the buffer.

**Amidation**—Apoferredoxin (5 mg, 7.2 μmoles) was dissolved in 2 ml of 0.1 M borate buffer, pH 8.5, and ethyl acetimidate·HCl (60 mg, 400 μmoles) was added and the pH was adjusted to 8.6 with 3 N NaOH. After 1 hour, another 50 mg of the reagent were added and the pH was adjusted to 8.5. An hour later, 50 mg of the reagent were added, the pH was adjusted to 8.4, and the reaction was allowed to proceed for another hour. The protein was reisolated by gel filtration on a Sephadex G-25 column equilibrated with 0.01 M sodium acetate and the column was developed with the same solution.

**Succinylation**—The procedure used was based on the method of Klotz (13). Apoferredoxin (5 mg, 13.2 μmoles) was dissolved in 13 ml of water and succinic anhydride (60 mg, 0.6 mmoles) was added with magnetic stirring in small increments to the solution over 1 hour at room temperature. The pH was maintained between 7 and 8 with addition of 3 N NaOH. The solution was allowed to stand for an additional 40 min and was then dialyzed overnight against 12 liters of 0.01 M sodium acetate. The determination of O-succinylation of hydroxyamino acid residues was carried out with the alkaline hydroxylamine reaction of Hestrin (14), whereas the procedure of Lipmann and Tuttle (15) was used for the estimation of active acyl groups with glycine methyl ester hydrochloride as standard.

**Synthesis of Aminoacylapoferredoxin**—Aminoacylapoferredoxins were synthesized from apofaredoxin by the method of Levy and Carpenter (16). Apoferredoxin (80 mg, 14.4 μmoles) was dissolved in 8 ml of 25:75 water-dimethylformamide medium, and the n-p-nitrophenyl ester of a BOC-amino acid (170 μmoles) and 15 μl of triethylamine were added. The reaction was allowed to proceed, with magnetic stirring, for 20 hours at room temperature. The solution was then passed over a Sephadex G-25 column (1.6 x 40 cm) to remove p-nitrophenol formed, and the cloudy, excluded fractions were pooled and extracted three times with equal volumes of ether. The aqueous portion was lyophilized under high vacuum. The lyophilized material was placed in a glass centrifuge tube and was dried thoroughly over P2O5 under high vacuum for 20 hours. The solid material was then dissolved in 2 to 3 ml of anhydrous trifluoroacetic acid and allowed to stand for 1 hour at room temperature. At the end of that time the solution was cooled in an ice bucket and the protein was precipitated with 4 ml of cooled ether. The precipitate was washed twice with ether and dried under nitrogen gas. The white solid material was dissolved in 5 ml of 0.1 M Tris-chloride buffer, pH 8.5, with a small amount of 1 M NaOH added to neutralize the amount of trifluoroacetic acid, and the solution was centrifuged and the supernatant was dialyzed against 0.01 M sodium acetate overnight. The yield was about 75 to 90%.

For the synthesis of glutamylapoferredoxin, saponification was carried out before the treatment with trifluoroacetic acid. After lyophilization, the solid material was dissolved in 10 ml of 0.034 M sodium carbonate buffer, pH 11.0, and allowed to stand 3 hours at room temperature (16). The protein was then precipitated by the addition of a solution of 30% trichloroacetic acid to bring the final concentration to 5% and the precipitate was washed with water and lyophilized.

**Carboxypeptidase A Treatment**—Apoferredoxin (50 mg) in 17 ml of 0.1 M Tris-chloride buffer, pH 7.0, was treated with 2 μg of carboxypeptidase A at room temperature for 7 hours. The enzyme solution was prepared by diluting 1 volume of the commercial stock suspension with 10 volumes of 10% LiCl and stirring for 1 hour at 0°. The concentration of enzyme was determined from absorbance at 278 nm (17). Ferredoxin was treated under the same conditions but was under anaerobic conditions to prevent the deterioration of ferredoxin. At the desired interval a 0.2-ml aliquot was withdrawn to 1 ml of 0.2 M sodium citrate buffer, pH 2.2, mixed, and centrifuged. The supernatant was then analyzed for amino acids on a Beckman-Spinco automatic amino acid analyzer, model 120 (18).

**Iodination**—Iodination was performed by the procedure of Gruen, Laskowski, and Scheraga (19). Apoferredoxin (41 mg, 7.4 μmoles) was dissolved in 5.5 ml of 0.5 M glycine-NaOH buffer, pH 9.5, and was iodinated over a period of 2.5 hours at 0° with 1 ml of 0.14 μl11-iodide solution (1 μmole = 60,000 cpm) (19). The protein was separated from the reagent by gel filtration on a Sephadex G-25 column previously equilibrated with 0.01 M Tris-chloride buffer, pH 8.5, and the column was developed with the same buffer.

**Preparation of BOC-apoferredoxin**—BOC-apoferredoxin was synthesized from apoferredoxin by the method of Levy and Carpenter (16). Apoferredoxin (70 mg, 11.3 μmoles) was dissolved in 10 ml of 25:75 water-dimethylformamide medium and 180 mg of BOC-azole and 25 μl of triethylamine were added. The solution was incubated for 7 hours at 40° and then the protein was reisolated by gel filtration on a Sephadex G-25 column (1.6 x 46 cm), equilibrated with water, and lyophilized. The yield was 87%.

**Regeneration of Apoferredoxin from BOC-apoferredoxin**—The condition used was the same as that used for the removal of the blocking group from the BOC-aminoacylapoferredoxins. The regenerated apoferredoxin was obtained in 74% yield.

**Determination of Free Amino Groups**—The degree of modification of the free amino groups was determined by measuring the

---

2 The abbreviations used are: BOC, t-butyloxycarbonyl group; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid).

4 We thank Dr. D. Levy, Mr. S. M. Shigii, and Professor F. H. Carpenter for making available to us the method for aminoacylation in aqueous dimethylformamide.
TABLE I
Extent of chemical modification of apoferredoxin

With the exception of acetyl derivatives, the extent of modification was determined by decrease in ninhydrin color. The percent of amino groups modified was calculated from the difference between the free amino groups present in the original and the modified proteins.

| Derivative                        | Amino group modified |
|----------------------------------|----------------------|
| Acetylapoferredoxin               | 85±                  |
| Acetylferredoxin reconstituted    | 82±                  |
| Acetimidoapoferredoxin            | 81                   |
| Acetimidoferredoxin reconstituted | 80                   |
| BOC-apoferredoxin                | 86                   |
| BOC-ferredoxin reconstituted      | 83                   |
| Succinylapoferredoxin             | 83                   |

* Moles of 14C-acetyl per mole of protein.

Fig. 1. The decrease in the biological activity of the reconstituted modified ferredoxin derivatives. The ferredoxin derivatives were assayed for the activity in the phosphoroclastic reaction. The activity of the ferredoxin derivative is expressed as the percent of that of the native ferredoxin but has been corrected for the absorbance due to the presence of an amount of unmodified ferredoxin (18% in acetyl-, 20% in acetimido-, and 17% in BOC-ferredoxin) in the reconstituted ferredoxin derivatives. A, native ferredoxin; B, acetimidoferredoxin; C, acetylferredoxin; D, BOC-ferredoxin.

RESULTS

Acetylferredoxin—The extent of the acetylation of ferredoxin and the apoferredoxins with 14C-acetic anhydride was determined from the radioactivity of the reisolated protein when native ferredoxin was subjected to acetylation with acetic anhydride. Although the NH2-terminal group of native ferredoxin does not react with acetic anhydride, apoferredoxin incorporated 0.87 μmole of 14C-acetyl group into 1 μmole of protein. Although the NH2-terminal group of native ferredoxin does not react with acetic anhydride, apoferredoxin incorporated 0.87 μmole of 14C-acetyl group into 1 μmole of protein (Table I). The acetylapoferredoxin was converted to acetylapoferredoxin_red (4) and this apoferredoxin_red derivative was used for reconstitution. The reconstituted acetylated ferredoxin contained 0.82 μmole of 14C-acetyl group per μmole of protein (Table I), and was obtained from apoferredoxin_red in a yield of 54%.

Acetylferredoxin had spectral properties identical with those of native ferredoxin. However, it was less stable than native ferredoxin as determined by its activity in the phosphoroclastic reaction (Fig. 1, Curve B). The decrease in activity followed first order kinetics with a half-time of 11 hours under the condi-
Native Methionyl-ferredoxin

Glutamyl-ferredoxin Lysyl-ferredoxin

Phenylalanyl-ferredoxin Glycyl-ferredoxin

Acetimido-ferredoxin Regenerated Ferredoxin

**FIG. 3.** Photomicrographs of crystals of *Clostridium acidi-urici* ferredoxin and its derivatives.

Under these conditions, native ferredoxin has a half-life of greater than 500 hours. In addition to the decrease of the biological activity, the absorption of acetylferredoxin at 390 nm was also found to decrease with time. The decay of the chromophore also followed first order kinetics (Fig. 2, Curve C).

Acetimidoferredoxin—Acetimidoferredoxin was obtained in crystalline form (Fig. 3). The ninhydrin reaction showed that approximately 20% of the amino groups of the acetimidopoferedoxin<sub>ox</sub> and reconstituted acetimidoferredoxin were free (Table I). The absorption spectrum of the reconstituted acetimidoferredoxin was indistinguishable from that of native ferredoxin. The derivative was 78% as active as native protein in the enzyme assay. This derivative is more stable in solution than acetylferredoxin, but it is less stable than solutions of native ferredoxin. The rate of loss of activity of the derivative in the enzyme test is shown in Fig. 1 (Curve A) and the rate of loss of its absorption at 390 nm is shown in Fig. 2 (Curve B). The half-time for the decay of activity was 132 hours.

**BOC-ferredoxin**—The absorption spectrum of the reconstituted BOC-ferredoxin was indistinguishable from native ferredoxin. It was about 78% as active as the native ferredoxin in the enzyme test. Approximately 14 and 17%, respectively, of the amino group of BOC-apoferredoxin<sub>ox</sub> and BOC-ferredoxin was unblocked as determined by the ninhydrin reaction (Table I). It was not as stable as acetylferredoxin. The half-time for the activity decay was about 4.5 hours. The decay of the activity and the decay of the absorption at 390 nm are shown in Fig. 1 (Curve C) and Fig. 2 (Curve D), respectively. The reconstituted BOC-ferredoxin was obtained from BOC-apoferredoxin<sub>ox</sub> and in about 43% yield. The BOC-ferredoxin was not obtained in crystalline form.

**Aminoacylferredoxin**—The following aminoacylferredoxin<sub>ox</sub> derivatives were synthesized: N-glycyl-, N-L-methionyl-, N-L-phenylalanyl-, N-L-lysyl-, and N-L-glutamyl-. They were obtained in about 75 to 90% yield. Ferredoxin derivatives could be reconstituted from these aminoacylapoferredoxin<sub>ox</sub> derivatives and were obtained in 45 to 54% yield. The aminoacylferredoxins were obtained in crystalline form. The photomicrographs of these crystals are shown in Fig. 3. Amino acid analysis of these reconstituted aminoacylferredoxin derivatives showed that a single amino acid residue had been added to the protein. The results of the amino acid analysis are shown in Table II. The addition of methionyl, phenylalanyl, or lysyl residues to the protein was apparent from the amino acid analysis because the native protein does not contain any residues of these amino acids.

The reconstituted aminoacylferredoxins were less active and

---

**Table II**

*Native and reconstituted* *Clostridium acidi-urici* ferredoxin and some derivatives

| Residue | Native | Methionyl- | Phenylalanine- | Glycine- | Glutamine- | Lysine-(Glu<sup>4</sup>, Ala<sup>8</sup>) |
|---------|--------|------------|---------------|----------|------------|----------------------------------|
| Asp     | 7.87   | 8.00       | 7.91          | 7.77     | 7.84       | 8.20                             |
| Thr     | 0.99   | 0.96       | 0.95          | 1.01     | 0.97       | 0.97                             |
| Ser     | 2.65   | 2.72       | 2.70          | 2.83     | 2.74       | 2.75                             |
| Glu     | 4.13   | 4.00       | 4.09          | 4.23     | 5.34       | 3.22                             |
| Pro     | 3.89   | 4.15       | 4.27          | 4.06     | 4.11       | 4.09                             |
| Gly     | 3.78   | 3.83       | 3.80          | 5.02     | 3.98       | 3.94                             |
| Ala     | 8.37   | 8.62       | 8.59          | 8.48     | 8.50       | 7.94                             |
| Cys     | 6.86   | 5.41       | 5.91          | 6.26     | 5.50       | 5.34                             |
| Val     | 5.22   | 5.20       | 5.43          | 5.95     | 5.70       | 5.41                             |
| Met     | 0.00   | 0.00       | 0.00          | 0.00     | 0.00       | 0.00                             |
| Ile     | 4.43   | 4.81       | 4.82          | 5.06     | 5.15       | 5.14                             |
| Tyr     | 1.73   | 1.86       | 1.92          | 1.77     | 1.66       | 1.65                             |
| Phe     | 0.00   | 0.00       | 0.00          | 0.00     | 0.00       | 0.00                             |
| Arg     | 0.93   | 0.90       | 0.90          | 1.01     | 0.82       | 0.90                             |
| Lys     | 0.00   | 0.00       | 0.00          | 0.00     | 0.00       | 0.96                             |

The addition of methionyl, phenylalanyl, or lysyl residues to the protein was apparent from the amino acid analysis because the native protein does not contain any residues of these amino acids. The reconstituted aminoacylferredoxins were less active and...
4992 Chemical Modification of Clostridial Ferredoxin

The ferredoxin derivatives were assayed for the activity in the phosphorolytic reaction. The activity of the ferredoxin derivative was expressed as the per cent of that of the native ferredoxin. A, glycylferredoxin; B, methionylferredoxin; C, lysylferredoxin; D, phenylalanylferredoxin; and E, glutamylferredoxin.

The decrease in A390 was expressed as per cent of the initial A390. The stability data show that the derivatives in which an amino acid residue with relatively small R groups were introduced were more stable than those with larger ones. Glutamyl- and lysyl-ferredoxins represent derivatives in which one negatively or one positively charged side chain, respectively, was added to the native protein. Glutamylferredoxin was 2.5 times less stable than lysylferredoxin.

The stability of phenylalanylferredoxin crystals was also examined. When stored as crystals suspended in ammonium sulfate (70% saturation) in 0.15 M Tris-chloride buffer, pH 7.4, at 4° under aerobic conditions, this derivative was found to retain 57.3% of the specific biological activity of the native protein after 4½ days of storage, a loss of 16% activity. This result indicates that ferredoxin is more stable when stored as a suspension of crystals than when stored in solution.

Regenerated Ferredoxin from BOC-apoferredoxin—Treatment of apoferredoxin with anhydrous trifluoroacetic acid resulted in the regeneration of an apoferredoxin which could be converted to ferredoxin and crystallized (Fig. 5). The reconstituted ferredoxin was indistinguishable from the native ferredoxin with respect to spectral properties, biological activity, and the stability as determined by its biological activity and absorption at 390 nm.

\[
\text{TABLE III}
\]

Summary of initial biological activity and stability of modified ferredoxin derivatives

| Ferredoxin derivative | Biological activity$^a$ | Stability (hrs) |
|----------------------|-------------------------|----------------|
| Native                | 100                     | 100            |
| Regenerated$^b$      | 100                     | 100            |
| Glycyl-              | 94                      | 53             |
| Acetyl-              | 86                      | 11             |
| Methionyl-           | 85                      | 31             |
| Acetimido            | 78                      | 132            |
| BOC-                 | 78                      | 4.5            |
| Des-(Ala$^{55}$, Gln$^{54}$)- | 77                  | 31             |
| Phenylalanyl-        | 68                      | 20             |
| Lysyl-               | 56                      | 43             |
| Glutamyl-            | 50                      | 20             |

$^a$ Expressed as the extrapolated initial activity.

$^b$ The reconstituted ferredoxin from the apoferredoxin regenerated from BOC-apoferredoxin.

stable than native ferredoxin. Figs. 4 and 5 show, respectively, the decay of the activity and absorption at 390 nm. The extrapolated initial activities as well as the half-time for the activity decay are summarized in Table III. These ferredoxin derivatives contained amino acid residues with side chains of varying charge and size. Glycyl-, methionyl-, and phenylalanylferredoxins retain the same net charge as native ferredoxin.

Fig. 4. The decrease in the biological activity of the reconstituted aminoacylferredoxins. The ferredoxin derivatives were assayed for the activity in the phosphorolytic reaction. The activity of the ferredoxin derivative was expressed as the per cent of that of the native ferredoxin. A, glycylferredoxin; B, methionylferredoxin; C, lysylferredoxin; D, phenylalanylferredoxin; and E, glutamylferredoxin.

Fig. 5. The decrease in the absorption at 390 nm of reconstituted aminoacylferredoxins. Reconstituted ferredoxin derivatives (initial A390 about 1) were kept in 0.1 M Tris-chloride buffer, pH 7.4, containing 0.1 M NaCl and the decrease in A390 was followed at 4° for 10 min at 9900 X g before measuring absorption. The decrease in A390 was expressed as per cent of the initial A390. A, native ferredoxin; B, glycylferredoxin; C, methionylferredoxin; D, phenylalanylferredoxin; and E, glutamylferredoxin.

Downloaded from http://www.jbc.org/ by guest on March 24, 2020
activity decay was 31 hours (Table III). The reconstituted des-(Ala$_{55}$, Gln$_{58}$)-ferredoxin contained 1 alanine and 1 glutamic acid residue less than native ferredoxin.

Native ferredoxin is not digested by carboxypeptidase A under conditions in which apoferredoxin is digested. This result suggests that the COOH-terminal amino acid residues of the native ferredoxin are not accessible to the carboxypeptidase A and might be buried in the native structure.

Succinylapoferredoxin—Succinylation of apoferredoxin with succinic anhydride resulted in the loss of 83% of the free amino group as determined by the ninhydrin reaction (Table I). The succinylapoferredoxin was converted to succinylapoferredoxine by succinic anhydride has been shown to react, not only with amino group but also with the side chain—OH groups of tyrosine, serine, and threonine residues in proteins (20), this inability of succinylapoferredoxin to form ferredoxin derivative might possibly be due to the succinylation of —OH groups of these amino acid residues in apoferredoxin. Succinylapoferredoxin was analyzed for the presence of O-succinyl groups. The result of the alkaline hydroxylamine reaction of Hestrin (14) showed the presence of approximately one O-succinyl per mole of protein whereas no active acyl group was found by the method of Lipmann and Tuttle (15). These results indicate that one —OH group of serine or threonine or a combination of these 2 amino acid residues in succinylapoferredoxin may have been succinylated.

Tetratidoapoferredoxin—Approximately 3.6 atoms of $^{131}$I were incorporated into 1 mole of apoferredoxin. This is slightly lower than 4, a value expected for iodination of the 2 tyrosine residues in the protein. A ferredoxin derivative could not be reconstituted from the tetratidoapoferredoxin. Sulphhydryl group analysis of the iodinated apoprotein by the DTNB method (21) after reduction with NaBH$_4$ in 8 M urea anaerobically showed that the protein had 7.1 sulphhydryl groups per mole of protein. This result indicates that the disulphides of apoferredoxin were not oxidized to the sulfonic acid level by iodination, although the formation of other oxo-derivatives that would be reduced to sulphhydryl level by borohydride was not eliminated, and that the inability of tetratidoapoferredoxin to reconstitute may most probably be attributed to the modification of the tyrosine residues.

When ferredoxin was iodinated under conditions identical with those used for iodination of apoferredoxin, it was bleached, indicating that iron and acid-labile sulfide were removed from the protein. Ferredoxin is apparently too labile to be subjected to iodination.

**Discussion**

The nature of the group used to modify the NH$_2$-terminal amino group had a marked effect on the stability of the ferredoxin derivatives that were obtained. These ferredoxin derivatives tend to lose their iron and acid-labile sulfide constituents at rates that are dependent on the modification introduced. No ferredoxin derivative formed was more stable in that respect than native ferredoxin. Although the rate of decay of the activity of the derivatives appears to be faster than the rate of decay of the absorption at 390 nm, interpretation of this observation is complicated by the fact that the released iron and sulfide form colored complexes. Acetylation or conversion to the BOC-derivative resulted in a net decrease of one positive charge of the protein. Although these derivatives, with no charge at the NH$_2$-terminal end, could be converted to the corresponding ferredoxin derivatives, the ferredoxin derivative formed showed markedly diminished stability compared to the native structure.

Amidination does not alter the net charge of the protein. The acetimidoferroferredoxin prepared from this apoferredoxin derivative was about 12 times more stable than acetylferredoxin. Since acetyl and acetimid groups are similar in size, the marked difference in the stabilities of these two ferredoxin derivatives must be attributed to the charge differences of these two derivatives, and indicates the importance of the NH$_2$-terminal positive charge in stabilizing the conformation of the protein. Since native ferredoxin does not react with reagents specific for free $\alpha$-amino groups, it appears that the positive charge of this amino group...
must be neutralized in the native protein by some counterion (carboxyl group) forming an ion pair. The presence of a positive charge at the NH₂-terminal end of aminoacylferredoxins apparently also increased the stability of these derivatives, although the positive charge is removed from its original position in native ferredoxin by one amino acid residue. Except for the glycylferredoxin, the other aminoacyl derivatives prepared carried blocking groups almost as bulky as BOC and would be expected to be no more stable than BOC-ferredoxin if only the steric effect of these blocking groups were operative. Their higher stability supports the view that the ion pair is a significant factor in conferring a stable conformation on the protein.

The steric effect of a blocking group, although not so profound as the charge effect, is apparent. For example, BOC-ferredoxin was less stable than acetylferredoxin, although neither derivative has a charge at the NH₂-terminal end; phenylalanyl and methionyl residues are larger than the glycy residue, and glycylferredoxin was found to be more stable than methionyl- and phenylalanylferredoxin.

The greater stability of lysylferredoxin compared to glutamylferredoxin suggests that the structural environment around the α-amino group at the NH₂-terminal end of the native protein probably could accommodate the positively charged side chain of a lysyl residue better than the negatively charged side chain of a glutamyl residue.

Iodoferredoxin represents the only example in these studies in which modification of nonterminal ends of the apoferrredoxin molecule was achieved. The failure to obtain a ferredoxin derivative from tetraiodoferredoxin might be due to the steric effect of the iodo groups, to the effect of the lowering of the pK of the phenolic hydroxyl groups of the tyrosine residues on possible hydrogen bonding involving this —OH, or to possible involvement of this —OH as a ligand of the iron atoms in the protein. One of these tyrosine residues occurs in position 2, next to the NH₂-terminal alanine. The observation that native ferredoxin does not react with several amino group reagents suggested that the NH₂-terminal alanine residues are “buried.” Thus it seems probable that the introduction of these 2 large iodine atoms on this tyrosine residue might interfere with the ability of the iodoferredoxin to form iodoferredoxin because of the steric effect of the iodo groups. However, further confirmation of this argument is required since the possible oxidation of the apoprotein disulfides by iodine to oxo-derivatives that apparently also increased the stability of these derivatives, although the positive charge is removed from its original position in native ferredoxin by one amino acid residue. Except for the glycylferredoxin, the other aminoacyl derivatives prepared carried blocking groups almost as bulky as BOC and would be expected to be no more stable than BOC-ferredoxin if only the steric effect of these blocking groups were operative. Their higher stability supports the view that the ion pair is a significant factor in conferring a stable conformation on the protein.

The steric effect of a blocking group, although not so profound as the charge effect, is apparent. For example, BOC-ferredoxin was less stable than acetylferredoxin, although neither derivative has a charge at the NH₂-terminal end; phenylalanyl and methionyl residues are larger than the glycy residue, and glycylferredoxin was found to be more stable than methionyl- and phenylalanylferredoxin.

The greater stability of lysylferredoxin compared to glutamylferredoxin suggests that the structural environment around the α-amino group at the NH₂-terminal end of the native protein probably could accommodate the positively charged side chain of a lysyl residue better than the negatively charged side chain of a glutamyl residue.

Iodoferredoxin represents the only example in these studies in which modification of nonterminal ends of the apoferrredoxin molecule was achieved. The failure to obtain a ferredoxin derivative from tetraiodoferredoxin might be due to the steric effect of the iodo groups, to the effect of the lowering of the pK of the phenolic hydroxyl groups of the tyrosine residues on possible hydrogen bonding involving this —OH, or to possible involvement of this —OH as a ligand of the iron atoms in the protein. One of these tyrosine residues occurs in position 2, next to the NH₂-terminal alanine. The observation that native ferredoxin does not react with several amino group reagents suggested that the NH₂-terminal alanine residues are “buried.” Thus it seems probable that the introduction of these 2 large iodine atoms on this tyrosine residue might interfere with the ability of the iodoferredoxin to form iodoferredoxin because of the steric effect of the iodo groups. However, further confirmation of this argument is required since the possible oxidation of the apoprotein disulfides by iodine to oxo-derivatives that can be reduced by borohydride but not by mercaptoethanol has not been eliminated.

The biological activities of the derivatives, calculated at time zero (Table III), particularly those with large blocking groups (lysyl-, phenylalanyl-, and glutamyl-), were lower than the native ferredoxin. Since the activity assays were performed at 37°, this lower activity might be caused by the thermal destruction of these derivatives in the assay and thus really reflect the instability of the protein rather than the inherent activity of the derivative in this assay. However, if this were the case, one would expect to find that those derivatives with the shortest t₁/₂ would also show the lowest biological activity at time zero. This was not observed. For example, the acetyl derivatives had high biological activity at zero time, but were very unstable. It seems, therefore, that the lower activity probably might be due to the lower affinity of these derivatives for the enzymes involved in the phosphoroclastic reaction.

REFERENCES
1. Tanaka, M., Nakashima, T., Benson, A. M., Mower, H. F., and Yasunobu, K. T., Biochemistry, 5, 1666 (1966).
2. Benson, A. M., Mower, H. F., and Yasunobu, K. T., Proc. Natl. Acad. Sci. U. S. A., 55, 1532 (1966).
3. Ball, S. C., Bolinger, R. E., and Cole, R. D., Biochemistry, 8, 2486 (1969).
4. Hong, J.-S., and Rabinoowitz, J. C., J. Biol. Chem., 245, 4982 (1970).
5. Hong, J.-S., and Rabinoowitz, J. C., Biochem. Biophys. Res. Commun., 29, 246 (1967).
6. Malkin, R., and Rabinoowitz, J. C., Biochem. Biophys. Res. Commun., 23, 822 (1966).
7. Hong, J.-S., and Rabinoowitz, J. C., J. Biol. Chem., 245, 4995 (1970).
8. Margolin, A., and Merrifield, R. B., Arch. Biochem. Biophys., 122, 748 (1967).
9. Rabinoowitz, J. C., in S. P. Colowick and N. O. Kaplan (Editors), Methods in enzymology, Vol. 6, Academic Press, New York, 1966, p. 763.
10. Loewenberg, W., Buchanan, B. R., and Rabinoowitz, J. C., J. Biol. Chem., 238, 3899 (1963).
11. Frerenkel-Comfort, H., in S. P. Colowick and N. O. Kaplan (Editors), Methods in enzymology, Vol. 4, Academic Press, New York, 1967, p. 247.
12. Wosky, L., and Singer, S. J., Biochemistry, 2, 104 (1963).
13. Klug, A. M., in C. H. W. Hirs (Editor), Methods in enzymology, Vol. 11, Academic Press, New York, 1967, p. 576.
14. Hestrin, S., J. Biol. Chem., 180, 249 (1949).
15. Lipmann, F., and Tuttle, L. C., J. Biol. Chem., 109, 21 (1945).
16. Levy, D., and Carpenter, F. H., Biochemistry, 6, 3559 (1967).
17. Vallee, B. L., and Neurath, H., J. Biol. Chem., 230, 64 (1960).
18. Spackman, D. H., Stein, W. H., and Moore, S., Anal. Chem., 30, 1190 (1958).
19. Gruen, L., Laszowski, M., Jr., and Scheraga, H. A., J. Biol. Chem., 234, 2050 (1959).
20. Gounalis, A. D., and Perlmann, G. E., J. Biol. Chem., 242, 2739 (1967).
21. Ferdinand, W., Stein, W. H., and Moore, S., J. Biol. Chem., 240, 1150 (1965).
The Effects of Chemical Modifications on the Reconstitution, Activity, and Stability of Clostridial Ferredoxin
J.-S. Hong and Jesse C. Rabinowitz

J. Biol. Chem. 1970, 245:4988-4994.

Access the most updated version of this article at http://www.jbc.org/content/245/19/4988

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/245/19/4988.full.html#ref-list-1