Immunological Properties and Conformational Differences Detected by Tritium-Hydrogen Exchange of Clostridial Ferredoxins and Apoferredoxins*

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SUMMARY

Antisera were prepared against the ferredoxins from Clostridium acidi-urici and Clostridium pasteurianum and characterized by quantitative precipitin and microcomplement fixation methods. The clostridial ferredoxins are thus the smallest naturally occurring polypeptides that have been shown to produce precipitating antibodies. The ferredoxin antibody of the immune rabbit serum is contained in the IgG fraction. The antiserum prepared against C. pasteurianum ferredoxin does not react with C. acidi-urici ferredoxin. Reconstituted ferredoxin is antigenically indistinguishable from the native ferredoxin as tested by microcomplement fixation. The antiserum react much more strongly with ferredoxin than with apoferredoxin. Interpretation of this observation with respect to showing the difference in the conformation of ferredoxin and apoferredoxin is limited because the possible role of the iron-sulfur chromophore of ferredoxin as an antigenic determinant is not known. However, experiments with the tritium-hydrogen exchange technique showed conformational differences between the polypeptide portions of apoferredoxin and ferredoxin. Differences between the conformations of the polypeptide chain of the oxidized and the reduced states of C. acidi-urici ferredoxin were also shown by the tritium-hydrogen exchange technique. The reduced state was found to have fewer sites for equilibration with tritium, and thus to have a conformation more compact than the oxidized state of the iron-sulfur protein. Ferredoxin reconstituted from the fully tritiated apoferredoxin was found to retain more replaceable hydrogen atoms than the native oxidized ferredoxin.

The unusual structure and chemical properties of clostridial ferredoxin and apoferredoxin prompted us to investigate the immunogenic activity of these proteins. Bacterial ferredoxins contain only 54 or 55 amino acid residues (1-4) and generally contain no histidine or tryptophan and only 1 or 2 residues of phenylalanine or tyrosine (5, 6). It is possible to remove the iron and sulfide moieties of the ferredoxin and obtain apoferredoxin, the polypeptide, free of these moieties. However, the structure of the iron-sulfur chromophore, a potential hapten, is not known. Since the amino acid sequences of Clostridium pasteurianum and Clostridium acidi-urici ferredoxin show a high degree of homology (1, 4), it was of interest to determine the cross-reactivity of the native proteins and apoproteins with antibodies produced in response to each protein. The availability of antibody for ferredoxin would also be useful as an additional specific method for the assay of ferredoxin. This would be particularly valuable since available assays are not sensitive (5).

Studies with ribonuclease (7, 8), polyalanyl rabbit immunoglobulin G (9, 10), and synthetic polypeptides (11) have shown that the steric conformation of the antigenic molecule, as well as its primary amino acid sequence, is an important factor in the determination of antigenic activity. We were therefore prompted to determine whether apoferredoxin could interact with the antibodies specific for ferredoxin, and to compare the antigenic activities of the native protein with that of the homologous apoproteins. However, conclusions with respect to the relative contributions of the two factors are limited in this case because the antigenic activity of the iron-sulfur chromophore of ferredoxin could not be tested directly.

We have investigated the nature of the relationships between the conformations of ferredoxin and apoferredoxin by measuring the rate of hydrogen exchange between the tritiated protein and solvent water first suggested by Hvidt and Lindergren Lang (12), with the Sephadex technique of Englander (13). C. acidi-urici apoferredoxin contains 50 peptide bond hydrogen atoms, 6 amide hydrogens, 6 hydroxyl hydrogen, 5 guanidinyl hydrogens, and 3 a-amino hydrogen atoms, or a total of 70 potential replaceable hydrogen atoms. Apoferredoxin and the oxidized and the chemically reduced forms of clostridial ferredoxins were equilibrated with tritiated water under identical conditions and the exchange out of tritium with solvent water was followed. The tritium-hydrogen exchange of the oxidized form of ferredoxin reconstituted from apoferredoxin that had been equilibrated with tritiated water was also determined.

EXPERIMENTAL PROCEDURE

Materials

Previously described procedures were used for the preparation of crystalline C. acidi-urici and C. pasteurianum ferredoxins.
Each reaction mixture contained 0.5 ml of rabbit antiserum and potassium phosphate buffer, pH 7.3.

II. Previously referred to as apoferredoxin III, and apoferredoxin, is serum by ultrafiltration and dialyzed against 0.9% NaCl-0.01 M NaCl.

Schechter, and Sela (20) followed by chromatography on DEAE-cellulose column as described by Levy and Sober (21). The IgG fraction was concentrated to the original volume of the antiserum by ultrafiltration and dialyzed against 0.9% NaCl-0.01 M NaCl. The reaction mixture, the column was washed with 40 ml of 0.1 M NaOH and redissolved in 1.2 ml of 0.1 M NaOH and the absorbance was measured at 280 nm in Zeiss PMQ II spectrophotometer at 25° with quartz cells of 1-cm light path. Microcomplement fixation assays were carried out as described by Levine and Van Vunakis (19).

Immunological Procedures

Antisera against C. pasteurianum and C. acidi-urici ferredoxins were prepared by immunization of male New Zealand White rabbits with the crystalline ferredoxins. The first injection, with 1.5 mg of ferredoxin in an emulsion with complete Freund’s adjuvant (Difco) in 1 ml, was distributed subcutaneously among four back sites. Two weeks later, the same dose with the same adjuvant was distributed among two toepad sites. Two weeks after that, the rabbits received an intravenous injection of 1.5 mg of ferredoxin in 1 ml of 0.9% NaCl solution through the marginal ear vein. Each injection was preceded by a test bleeding.

Blood taken 2 weeks after the intravenous injection showed no antibodies by the microcomplement fixation method of Levine and Van Vunakis (19). The rabbits were rested for 4 months and then the whole procedure described above was repeated with 4 to 7 mg of ferredoxin for each injection.

The rabbits finally received two additional intravenous injections on the alternating days following the last injection. Blood was collected 1 week after the last injection. Sera were stored at -15°. Two rabbits were used for the production of antibodies against C. pasteurianum ferredoxin and one for C. pasteurianum ferredoxin. Rabbit immuno-γ-globulin was prepared by ammonium sulfate fractionation as described by Schechter, Schechter, and Sela (20) followed by chromatography on DEAE-cellulose column as described by Levy and Sober (21). The IgG fraction was concentrated to the original volume of the antiserum by ultrafiltration and dialyzed against 0.9% NaCl-0.01 M potassium phosphate buffer, pH 7.3.

Quantitative precipitin reactions were carried out as follows. Each reaction mixture contained 0.5 ml of rabbit antiserum and an increasing amount of Fe-ferredoxin in 0.033 M Tris-chloride buffer, pH 7.4, containing 0.12 M NaCl, and was made up to a final volume of 1.0 ml with the same buffer. The reaction mixture was incubated for 1 hour at 37° and then for 18 hours at 4°. The precipitates were collected by centrifugation and were washed three times at 0° each with 0.7 ml of the Tris-chloride buffer, pH 7.4, containing 0.12 M NaCl and redissolved in 1.2 ml of 0.1 M NaOH and the absorbance was measured at 280 nm in Zeiss PMQ II spectrophotometer at 25° with quartz cells of 1-cm light path. Microcomplement fixation assays were carried out as described by Levine and Van Vunakis (19).

Equilibration with Tritiated Water

Oxidized Ferredoxin—Tritiated water (30 mCi in 0.3 ml) was added to the native C. acidi-urici ferredoxin (30 mg) in 8 ml of 0.1 M Tris-chloride buffer, pH 8.0, in a Thunberg tube and the tube was evacuated for 10 min with an aspirator and allowed to stand anaerobically for 1 hour at room temperature and then 40 hours at 4°.

Reduced Ferredoxin Native C. acidi-urici ferredoxin (30 mg) in 8 ml of 0.1 M Tris-chloride buffer, pH 8.0, in a Thunberg tube was evacuated for 10 min with an aspirator, reduced by addition of 50 mg of sodium hydrosulfite, and evacuated for 10 min. Tritiated water (30 mCi in 0.3 ml) was added to this solution and the tube was evacuated for 10 min and allowed to stand anaerobically for 1 hour at room temperature and then 40 hours at 4°.

Reconstitution of Ferredoxin from Tritated Apoferredoxin

Apoferredoxin_red from C. acidi-urici (21 mg) in 10 ml of 0.1 M Tris-chloride buffer, pH 8.0, containing 0.05 M 2-mercaptoethanol was incubated with 2 ml per ml of tritiated water for 1 hour at room temperature and then 40 hours at 4°. For reconstitution of ferredoxin from this tritiated apoferredoxin_red, solid ferrous ammonium sulfate (7.8 mg) and sodium sulfide:9 HzO (4.8 mg) were added and the mixture was allowed to stand at room temperature for 10 min. The reconstituted ferredoxin was isolated by chromatography on a DEAE-cellulose column (1.4 × 10 cm) previously equilibrated with 0.5 M potassium phosphate buffer, pH 6.5, and washed before use. After applying the reaction mixture, the column was washed with 40 ml of 0.01 M Tris-chloride buffer, pH 7.4, containing 0.225 M NaCl, and the reconstituted ferredoxin was eluted with 0.1 M Tris-chloride buffer, pH 7.4, containing 0.5 M NaCl. The eluate (4 ml) was passed over a Sephadex G-25 column (2.4 × 10 cm) equilibrated with 0.01 M Tris-chloride buffer, pH 8.0, containing 0.05 M NaCl. It required about 30 min to recover the ferredoxin from the reconstitution mixture by this chromatographic procedure.

Removal of Tritiated Water by Gel Filtration

The rate of loss of protein-bound tritium from ferredoxin samples was followed by the technique of Englander (13). The

1 Apoferredoxin_red stands for the reduced apoferredoxin previously referred to as apoferredoxin III, and apoferredoxin_red is the oxidized apoferredoxin previously referred to as apoferredoxin II.
one-column procedure was used to measure the exchange rate up to 40 min. For the longer time periods, the two-column procedure was used. In the two-column experiments, a known volume of the eluate from the first gel filtration step was mixed with a known amount of nonradioactive ferredoxin in the same buffer to increase the concentration of protein for the second gel filtration. The fraction of the absorbance due to the tritiated ferredoxin was calculated and used to normalize the specific activity of the effluent protein from the second column.

All Sephadex G-25 columns (2.4 x 9 cm) were previously equilibrated at 4°C with 0.01 M Tris-chloride buffer, pH 8.0, containing 0.05 M NaCl. For the one-column procedure a 1-ml sample was used, and the elution required approximately 2 min. For the two-column procedure 3 ml of a sample were first passed through the first column and the first two-thirds of the effluent protein was pooled, mixed with a known amount of nonradioactive ferredoxin, and incubated under the conditions described. Aliquots (1 ml) were then passed through a second column to remove unbound tritium. The concentrations of ferredoxin in the eluted fractions were determined spectrophotometrically. The radioactivity of 0.1-ml aliquots of the eluate was determined.

The number of unexchanged hydrogen atoms per molecule of ferredoxin was calculated from the specific activity of effluent protein with the formula given by Englander (13).

RESULTS

Homogeneity of System—Immunodiffusion experiments against a crude cell-free extract of C. acidii-urici showed that the antiserum from a rabbit immunized with C. acidii-urici ferredoxin was immunologically impure since in addition to a precipitin line corresponding to that observed with the pure homologous ferredoxin at least one weaker line was formed (Fig. 1). Despite the heterogeneity of the antiserum, presumably due to some impurity present in the ferredoxin samples or formed from the ferredoxin in the immunization procedure, the immunochemical system was considered to be homogeneous since a single precipitin line was observed by immunodiffusion against the homologous crystalline ferredoxin (Fig. 1A). In addition, the observation of a single precipitin curve in the quantitative precipitin reaction and a single complement fixation curve, shown below, established that the immunochemical reaction being measured in this work is ferredoxin-antiferredoxin.

**Immunological Activity of C. acidii-urici Ferredoxin—Antiserum from a rabbit immunized with C. acidii-urici ferredoxin was tested in the precipitin reaction with C. acidii-urici 57Fe-ferredoxin and the apoferredoxin, derived from it. A single precipitin curve was obtained with C. acidii-urici ferredoxin and the 57Fe was found to be associated with the precipitates (Fig. 2, A and B). No precipitin curve was observed with the apoferredoxin, (Fig. 2C). Normal serum collected from an unimmunized rabbit was also tested by this technique with C. acidii-urici ferredoxin. No precipitin was observed (Fig. 2, D and E). Immunodiffusion experiments also showed that no precipitin was observed with apoferredoxin (Fig. 1).

These C. acidii-urici ferredoxin antisera were also tested by microcomplement fixation with ferredoxin and apoferredoxin from C. acidii-urici. The results obtained are shown in Fig. 3. Whereas C. acidii-urici ferredoxin fixed complement at 1:800 dilution of the antiserum, the apoferredoxin derived from it did not react at this dilution. If the antiserum was used at higher concentrations (1:100, 1:200 dilution), complement-fixing activity of the apoferredoxin could be observed. This result indicates that the ferredoxin reacted more effectively than apoferredoxin with antiserum to C. acidii-urici ferredoxin.

Rabbits were also injected with C. acidii-urici apoferredoxin by the same procedure as described for immunization with ferredoxin. No reaction could be detected with the sera from

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**Fig. 1.** Gel diffusion of C. acidii-urici ferredoxin, apoferredoxin, and an extract of C. acidii-urici against C. acidii-urici ferredoxin antiserum. A, Wells 1 through 4 contained 0.85, 1.70, 2.55, and 3.4 μg of ferredoxin, respectively. B, Wells 1 through 3 contained 2.6, 3.4, and 5.1 μg of apoferredoxin, respectively. Well 4 contained 3.4 μg of ferredoxin. C, Wells 1 and 4 contained 3.4 μg of ferredoxin. Well 2 contained 15 μl of a crude extract of C. acidii-urici equivalent to 0.255 mg of protein. The ferredoxin content of this sample, as determined by enzymatic assay, was 2.55 μg. Well 3 contained 3.4 μg of apoferredoxin.

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**Fig. 2.** Quantitative precipitin reactions of C. acidii-urici ferredoxin and apoferredoxin with antiserum to C. acidii-urici ferredoxin. Quantitative precipitin reactions were performed as described under "Experimental Procedure." The C. acidii-urici 57Fe-ferredoxin had a specific activity of 2.25 x 10⁶ cpm per mg. Control serum was collected from unimmunized rabbits. C. acidii-urici ferredoxin: A, O--O, Asso; B, ---, radioactivity. C. acidii-urici apoferredoxin: C, □--□, A₁₀₀; D, □--□, A₂₀₀. Control serum with C. acidii-urici ferredoxin: E, □--□, radioactivity.
Immunochemical Activity of C. pasteurianum Ferredoxin—

Similar results were also obtained with the antiserum from a rabbit immunized with C. pasteurianum ferredoxin. When tested by the precipitin reaction with \(^{59}\)Fe-ferredoxin from C. pasteurianum and C. acidi-urici, a single precipitin curve was observed with C. pasteurianum ferredoxin and the \(^{59}\)Fe was associated with the precipitates (Fig. 4, A and B) but no precipitin was observed with C. acidi-urici ferredoxin (Fig. 4, D and E). In the test by microcomplement fixation, C. pasteurianum ferredoxin fixed complement at 1:1000 dilution of the antiserum but the apoferredoxin derived from it did not react at this dilution (Fig. 5). However, if the antiserum was used at higher concentration (1:250 dilution), complement-fixing activity of the apoferredoxin could be observed.

Antiserum IgG—When IgG was isolated and purified from the antiserum specific to C. acidi-urici ferredoxin and tested by the microcomplement fixation with C. acidi-urici ferredoxin, the antigen fixed complement at 1:600 dilution of the IgG (Fig. 6A). The interaction of IgG and the antigen indicates that the antibody molecules specific to ferredoxin are contained in immuno-\(\gamma\)-globulin (IgG) fraction.

Identical Conformation of Native and Reconstituted Ferredoxin—

Ferredoxin reconstituted from apoferredoxins has been shown to be indistinguishable from native ferredoxin with respect to spectral properties, iron and sulfide content, and enzymic activity (17, 20). When tested by the microcomplement fixation test...
nique with IgG specific to C. acidi-urici ferredoxin both the reconstituted and the native ferredoxin from C. acidi-urici fixed complement to the same extent (Fig. 6, A and B). This result indicates that both the native and the reconstituted ferredoxins have identical conformations.

**Tritium-Hydrogen Exchange**—The exchange curve of apoferredoxin\(_{\text{red}}\) at pH 8.0 and 4° is shown in Fig. 7 (curve D). Only a very small amount of tritium was retained in apoferredoxin\(_{\text{red}}\). In contrast to this result, ferredoxin was found to retain significant amounts of tritium. The exchange curve for the native oxidized ferredoxin at pH 8.0 and 4° is shown in Fig. 7 (Curve B). Two separate experiments were carried out under identical conditions and the results show good reproducibility. Because of the instability of ferredoxin in medium with pH values below 7 or higher than 8.5, the exchange was carried out only at pH 8.0. Analysis of the exchange curve according to the procedure described by Englander (13) shows that there are about 27 exchangeable hydrogen atoms, excluding those extremely rapidly exchanging species with 4\( ^{1} \) values of less than 20 min.

The exchange curve obtained with reduced ferredoxin prepared as described under “Experimental Procedure” and equilibrated with tritiated water under the same conditions as used for the oxidized ferredoxin and the apoferredoxin\(_{\text{red}}\) is shown in Fig. 7 (Curve C). Although we refer to this protein as “reduced” ferredoxin, the protein was automatically reoxidized in the course of the analysis since no reducing agent was added to the buffer used for gel filtration. The exchange curve parallels that obtained with the oxidized ferredoxin; however, the protein retained only 17 exchangeable hydrogen atoms, a significantly smaller number than observed for the oxidized ferredoxin.

The fully tritiated apoferredoxin\(_{\text{red}}\) was converted to ferredoxin by the addition of an iron salt and sulfide and the ferredoxin formed was purified by chromatography on DEAE-cellulose. The tritiated solvent was separated from the reconstituted ferredoxin which was tightly adsorbed on the top of the column. The reconstituted ferredoxin eluted from the column was passed through a Sephadex G-25 column so that the protein could exchange in the same buffer as used for the exchanges of other ferredoxin samples. The exchange of the reconstituted oxidized ferredoxin at pH 8.0 and 4° is shown in Fig. 7 (Curve A). The exchange curve parallels that obtained with the oxidized ferredoxin; however, in this case the protein was observed to retain 35 hydrogen atoms, the largest number among the three protein species examined.

**DISCUSSION**

Clostridial ferredoxins were shown to have immunogenic activity for rabbits as determined by microcomplement fixation or by precipitin formation. The clostridial ferredoxins are thus the smallest naturally occurring polypeptides that have been shown to produce precipitating antibodies.

Attempts to elicit antibodies against apoferredoxin were unsuccessful by this procedure. The antigenic activity of clostridial ferredoxins, however, is poor, as is evident from the lack of complement fixation at serum dilutions greater than 1:500 to 1:1000. This relatively poor antigenicity is not surprising in view of the relatively small molecular weight of these proteins and the fact that the unmodified protein was used in immunization. Nevertheless, the microcomplement fixation method offers an assay that is 10 to 20 times more sensitive with respect to ferredoxin than the standard enzymic assay with the phospho-elastic reaction (5). The immunological test requires only about 0.03 \( \mu \)g of protein compared to 0.3 to 9 \( \mu \)g needed for the phospho-elastic reaction (5).

The antibodies that were formed in response to C. pasteurianum and C. acidi-urici ferredoxins are specific and do not cross-react. These two ferredoxins differ in 14 of the 55 amino acid residues in their sequences. These observations serve to confirm the importance of the primary amino acid sequence as one of the factors controlling the antigenic determinants of a protein molecule, since even though the amino acid sequences of these two ferredoxins differ it seems likely that the conformations of these two clostridial ferredoxins are very similar, at least with respect to the structure involving the iron, sulfide, and cysteine moieties of the proteins.

The apoferredoxin\(_{\text{red}}\) of both C. acidi-urici and C. pasteurianum failed to form a precipitin with antisera to their respective ferredoxins. The apoproteins did show a reaction in the microcomplement fixation test with the antiserum to their respective ferredoxins, but it was necessary to use concentrations of antiserum 4- to 8-fold higher than was necessary when using the homologous ferredoxin. The experiments with the tritium-hydrogen exchange of apoferredoxin and ferredoxin clearly indicate a difference in the conformation of the polypeptide portion of these molecules, since the apoprotein exhibited instantaneous exchange of the hydrogen atoms characteristic of “structureless” polypeptides, whereas the slower exchange rate of the hydrogen atoms of ferredoxin is indicative of the inaccessibility of hydrogen atoms because of their bonding in a specific conformation that is not freely accessible to the aqueous medium.

Although the tritium-hydrogen exchange data provide evidence that the conformations of apoferredoxin and ferredoxin differ, it is not possible to conclude that the conformational differences are alone responsible for the difference in immunological activity, since the role of the iron-sulfur chromophore present in the native protein as an immunological determinant has not been determined. It is unlikely that the weak immunological reaction that was observed with apoferredoxin could be due to the formation of ferredoxin from the apoprotein by chemical reconstitution because the conditions of the microcomplement fixation test are not favorable to reconstitution. The observed reaction might be due to the presence of a small amount of...
antibody in the ferredoxin antiserum specific toward apoferredoxin, since some apoferredoxin is probably formed from the ferredoxin under the conditions of the immunization. Although apoferredoxin was not found to be antigenic, it is possible that apoferredoxin formed under the immunization conditions could differ in chemical structure from the apoferredoxin, and could be antigenic.

Our observations are not consistent with the observations reported by Nitz et al. (24) that apoferredoxin reacts more strongly than does native ferredoxin with antiferredoxin antibodies. In our experiments with the quantitative precipitin reaction, no precipitation was observed between apoferredoxin and antiferredoxin antibodies. The C. pasteurianum ferredoxin used by Nitz et al. (24) was not pure as judged by the reported A500/A280 of 0.72 to 0.76 compared to the value of 0.81 for the pure material (14). It is therefore possible that the immunochemical reaction measured by these investigators is not that of ferredoxin and antiferredoxin.

A number of previous studies have described changes in the physical and chemical properties of the chromophore of clostridial ferredoxins that were induced by reduction of the native oxidized molecule (25-27). Chemically reduced ferredoxin from Clostridium aciditipienti was shown to be less reactive toward phenanthroline than native ferredoxin (25). Similar results were also observed with C. pasteurianum ferredoxin (26). Optical rotatory dispersion studies showed marked changes in the asymmetry of the chromophore upon reduction of ferredoxin by hydrosulfite (27). These studies, however, did not provide information concerning the extent of the possible alterations of the conformation of the peptide induced by oxidation-reduction.

The effect of oxidation and reduction of the ferredoxin on the conformation of the polypeptide portion of the molecule was examined here with the use of the tritium-hydrogen exchange technique. This has been used as a general but sensitive probe of protein conformation. Both the pH of the medium and the temperature have been shown to affect the number of protein hydrogen atoms that occur in the particular classes that can be recognized by this technique (28). The effect of pH was not investigated in the studies on ferredoxin reported here, and all experiments were carried out at pH 8.0 at which ferredoxin shows optimal stability. Even at this relatively high pH value, significant numbers of hydrogen atoms with relatively slow exchange rates were observed with both the oxidized and reduced ferredoxins.

The results obtained in the present studies show that the exchange curves of oxidized and reduced C. aciditipienti ferredoxin differ considerably and therefore indicate that the conformations of these states of the protein differ. Under the experimental conditions used, the chemically reduced ferredoxin is recovered in the oxidized form when it is passed over a column of Sephadex G-25 and is separated from the reducing agent. Thus, the reduced ferredoxin is recovered in a "reoxidized" form, and the hydrogen-tritium exchange of reduced ferredoxin also has superimposed upon it the exchange of the oxidized form of the protein.

Examination of the tritium-hydrogen exchange curves indicates that the three ferredoxin samples examined—the native oxidized, the reduced, and the reconstituted ferredoxins—all have similar hydrogen-tritium exchange curves. However, oxidized ferredoxin was found to have a larger number of slowly exchangeable hydrogen atoms than the reduced ferredoxin. This observation suggests that the reduced state has a more compact structure than the oxidized state of the protein since it has fewer sites for equilibration with tritium.

When ferredoxin is reconstituted from the fully tritiated apoferredoxin by the addition of an iron salt and sulfide, a drastic change in the conformation of apoferredoxin would be expected to occur since 8 moles each of iron and sulfide become incorporated into the protein in this process. The ferredoxin formed in the reconstitution reaction from fully tritiated apoferredoxin was shown to contain the most slowly exchangeable hydrogen atoms of any of the proteins examined. This indicates that a substantial portion of the replaceable hydrogen atoms is "buried" or participates in hydrogen bonding in the functional protein (or both).

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