The Reactivity of Clostridial Ferredoxin with Iron Chelating Agents and 5,5′-Dithiobis-2-nitrobenzoic Acid*

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ABSTRACT: The reaction of *Clostridium acidi-urici* ferredoxin with the ferrous chelating agent *o*-phenanthroline) is relatively slow under anaerobic conditions. After either chemical or enzymatic reduction of the protein, there is a rapid reaction of one of the seven to eight iron atoms with the chelating agent but the reaction of the remaining iron is inhibited. In the presence of high concentrations of urea or guanidine hydrochloride and aerobic conditions, all the iron in the protein reacts with *o*-phenanthroline and the rate follows first-order kinetics. Only 65–80% of the iron reacts under anaerobic conditions in the presence of these denaturants, but the remaining iron reacts upon aeration of the solution. Ferredoxin undergoes gradual degradation in the presence of 6.4 M urea or 4 M guanidine hydrochloride under aerobic conditions, but the protein is relatively stable in these denaturants under anaerobic conditions. Native, enzymatically reduced, or chemically reduced ferredoxin does not react with the ferric chelating agent, Tiron. In 4 M guanidine hydrochloride under aerobic conditions, 8 moles of iron/mole of protein reacts with Tiron and the reaction rate follows first-order kinetics. Under anaerobic conditions, however, only approximately half the iron in the protein reacts with Tiron. Native ferredoxin does not react with the sulfhydryl reagent 5,5′-dithiobis-(2-nitrobenzoic acid (DTNB), and there is only a slight reaction in the presence of 6.4 M urea. In 4 M guanidine hydrochloride, under either anaerobic or aerobic conditions, approximately 14 moles of DTNB react/mole of ferredoxin. This reaction is due exclusively to the inorganic sulfide in ferredoxin. The reaction of both the inorganic sulfide and the cysteine residue in ferredoxin with DTNB occurs in the presence of 4 M guanidine hydrochloride and EDTA under anaerobic conditions.

The requirement for EDTA suggests that the ferric iron atoms are involved in sulfhydryl group oxidation. When DTNB and a ferrous chelating agent are added to ferredoxin in 4 M guanidine hydrochloride under anaerobic conditions, 14 moles of DTNB react/mole of ferredoxin and all the iron appears as the ferrous complex. This indicates that the over-all reaction involves the reduction of ferric iron and the oxidation of an equivalent amount of the protein sulfhydryl groups. If DTNB is allowed to react with ferredoxin under conditions in which 14 moles of DTNB react with the protein, and a ferrous or ferric iron chelating agent is then added to the protein, only 70% of the iron in the protein reacts with either iron chelating agent and the remainder of the iron is no longer reactive even under aerobic conditions. This indicates that after the inorganic sulfide of ferredoxin has reacted with DTNB, the protein no longer is oxygen sensitive and that the site of oxygen sensitivity in ferredoxin is the inorganic sulfide moiety in the protein.

Although clostridial ferredoxin was identified as an iron protein at the time of its initial discovery (Mortenson *et al.*, 1962), the nature of the iron bonding in the native protein is not yet known. The inability of the iron of native ferredoxin to exchange with added [^{59}Fe]-citrate (Lovenberg *et al.*, 1963) suggested that the iron is not present in simple ionic bonding. Other experiments indicated the possibility that the iron was bonded to the cysteine residues in the peptide chain (Lovenberg *et al.*, 1963).

Some of the characteristics of the reaction of ferredoxin with α,α'-dipyridyl at pH 5.4 have been described by Malkin and Rabinowitz (1966a) and by Gillard *et al.* (1965). The latter investigators subsequently prepared and partially characterized "iron-depleted" ferredoxins (Gillard *et al.*, 1966). These studies demonstrated the essential role of the iron for the biological activity of the protein and also indicated that not all the iron in the protein is required for enzymic activity.

Ferredoxin contains a form of sulfur that has been termed "inorganic sulfide" in addition to the sulfur found in the cysteine residues present in the protein. The inorganic sulfide is released as H_2S when the protein is treated with acid or iron chelating agents (Lovenberg *et al.*, 1963; Malkin and Rabinowitz, 1966a,b).

Analysis of ferredoxin with *o*-phenanthroline after

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hydrolysis of the native protein with acid shows the presence of 7–8 moles of iron in the ferrous state (Lovenberg *et al.*, 1963). However, if the protein is first treated with a mercurial, only four atoms are obtained in the ferrous state (Sobel and Lovenberg, 1966; Palmer *et al.*, 1966). These results suggest that the sulfhydryl groups of the native protein are involved in the reduction of the ferric iron(III) to ferrous iron(II) during the chemical analysis.

The interpretation of these results with respect to the oxidation state of the iron in the native protein is subject to significant objections. Not only are the chemical methods difficult to carry out in the complex mixtures analyzed, but the ligands of the metal in native ferredoxin are still not known, and may involve degrees of electron sharing that would make assignment of normal oxidation states impossible. It should therefore be recognized that the analyses of the amounts of ferric, ferrous, sulfhydryl, and disulfide reported in this investigation are those obtained after various chemical treatments have "fixed" the electrons of the molecule among the components being measured, and although this must reflect the over-all electron balance, it may not represent the state of the native protein.

In this report, the characteristics of the reaction of clostridial ferredoxin with ferrous and ferric chelating agents under a variety of conditions, and the effect of these reactions on the sulfide and sulfhydryl groups of ferredoxin, are described.

Experimental Procedures

Materials. The following reagents were purchased from the indicated commercial sources: 5,5′-dithiobis-2-nitrobenzoic acid (DTNB)[1] (Aldrich Chemical Co.), *o*-phenanthroline (G. F. Smith Chemical Co.), sodium catecholdisulfonate (Tiron) (K & K Laboratories), and sulfonated bathophenanthroline, sodium salt (G. F. Smith Chemical Co.). Guanidine hydrochloride was purchased from Eastman Organic Chemicals and recrystallized from methanol before use. Urea was purchased from the Fisher Chemical Co. and recrystallized from water. A urea solution (8 M) was then prepared and deionized on a column of mixed-bed Resin AG-501-X8 (Bio-Rad Laboratories). The solution was stored at 4°.

Clostridium acidi-urici was grown with uric acid as the carbon and nitrogen source, as described by Rabinowitz (1963). Ferredoxin from *C. acidi-urici* was isolated and crystallized according to the procedure of Buchanan *et al.* (1963). The ferredoxin used in these studies had an A_{390}/A_{280} of 0.78 and samples with a ratio of less than 0.75 were repurified before use.

Methods. Iron was determined with *o*-phenanthroline by the procedure described by Lovenberg *et al.* (1963).

Inorganic sulfide was determined by the procedure of Lovenberg *et al.* (1963), except that the *N,N*-dimethylphenylenediamine hydrochloride reagent was prepared in 5.5 N HCl and the $FeCl_3$ reagent was prepared in 1.2 N HCl.

Analysis of the ferric–Tiron complex in the presence of DTNB was carried out by measuring the extinction at 540 mμ, where there was no color from the DTNB reaction. The millimolar extinction coefficients for ferric–Tiron complex at 540 and 412 mμ were found to be 4.15 and 2.62, respectively. The ferrous–sulfonated bathophenanthroline complex was found to have millimolar extinction coefficients at 535 and 412 mμ of 22.1 and 6.20, respectively. All calculations on the sulfhydryl content of samples in the presence of either iron reagent were made after corrections for the absorption at 412 mμ due to the respective iron complex with these experimentally determined extinction coefficients.

Results

The Reaction of Clostridial Ferredoxin with o-Phenanthroline. In contrast to the behavior of acid-treated ferredoxin (Lovenberg *et al.*, 1963), native *C. acidi-urici* ferredoxin reacts slowly with *o*-phenanthroline under either aerobic or anaerobic conditions. The reactivity of ferredoxin toward the ferrous chelating agent, *o*-phenanthroline, was determined under various conditions by measuring the increase in extinction at 512 mμ caused by the formation of ferrous–*o*-phenanthroline complex. The time course of the reaction of *C. acidi-urici* ferredoxin with *o*-phenanthroline under anaerobic conditions is shown in Figure 1. The reaction rate is zero order for at least the first 30 min after the addition of the chelating agent (curve A). The rate constant in this experiment was 0.272 mμatom of iron reacting/min. If ferredoxin is chemically reduced with sodium hydrosulfite (curve C) or is enzymatically reduced with a hydrogenase preparation from *Clostridium pasteurianum* in the presence of hydrogen gas (curve B), and *o*-phenanthroline then is added, there is an initial rapid reaction in which approximately 15% of the total iron present reacts with the chelating agent. This is equivalent to the reaction of approximately one atom of ferrous iron per mole of protein. This initial reaction is followed by a very slow reaction in the case of the chemically reduced sample (curve C). The difference in the slow rate between the chemically and enzymatically reduced samples is of questionable significance since the reaction of the enzymatically reduced sample has been corrected for the reaction of the chelating agent with the hydrogenase preparation alone, and this correction introduces some error in these experimental values.

The reaction of *C. acidi-urici* ferredoxin with *o*-phenanthroline is markedly stimulated in the presence of guanidine hydrochloride or urea. Sodium dodecyl sulfate at concentrations up to 5% had no effect on the reactivity of the iron toward the ferrous chelating agent. The time course and extent of the reaction in urea and guanidine hydrochloride under aerobic conditions are shown in Figure 2. All the iron in the protein reacted with the chelating agent in the presence

[1] Abbreviation used: DTNB, 5,5′-dithiobis-2-nitrobenzoic acid.

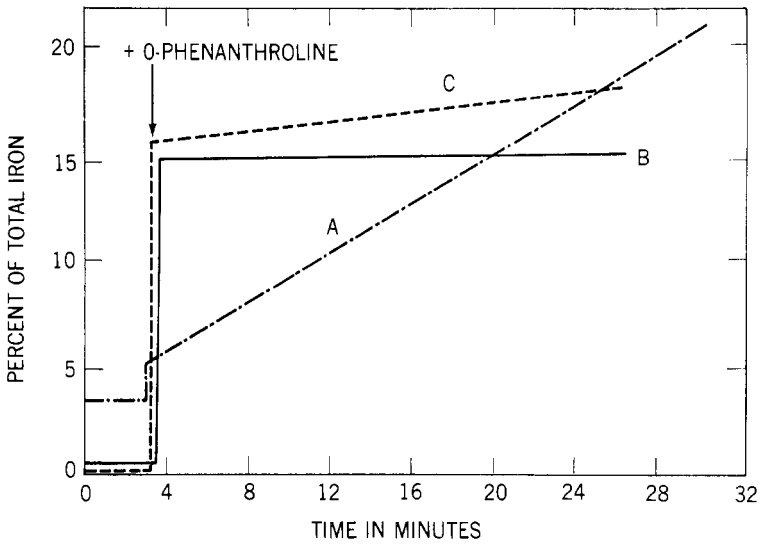

FIGURE 1: The reaction of native and reduced ferredoxins with *o*-phenanthroline under anaerobic conditions. Reaction mixtures contained 18.7 μg of *C. acidi-urici* ferredoxin equivalent to 24.3 mμatoms of iron, 0.1 M Tris-HCl (pH 7.4), and 0.73 mM *o*-phenanthroline in a final volume of 1.0 ml. The reaction mixtures, in 1.5-ml cuvets stoppered with silicone rubber injection gaskets, were flushed with hydrogen gas for 10 min at room temperature prior to the addition of the protein. The protein was added with a microsyringe through the injection gasket. The reaction was started by the addition of the iron chelating agent and the absorbance change at 512 mμ was followed in the Cary Model 14 spectrophotometer. Hydrogenase was used for the enzymic reduction of ferredoxin. The ferredoxin-free "clastic system" of *C. pasteurianum* described by Lovenberg *et al.* (1963) was used as a source of the enzyme. The preparation was kept frozen in liquid nitrogen, rather than as the lyophilized powder previously described. In this experiment, 2 μl of a preparation containing 32 mg of protein/ml was used as a source of the enzyme. The absorbance change for the enzymatically reduced sample is corrected for the reaction of *o*-phenanthroline with the hydrogenase preparation under identical conditions. For chemical reduction, ferredoxin was reduced with 0.2 mg of $Na_2S_2O_4$ prepared in degassed water. All calculations were based on a millimolar extinction coefficient of 10.5 for the *o*-phenanthroline–iron(II) complex under these conditions. (A) Native ferredoxin (– · – · –); (B) enzymatically reduced ferredoxin (———); (C) chemically reduced ferredoxin (– – – –).

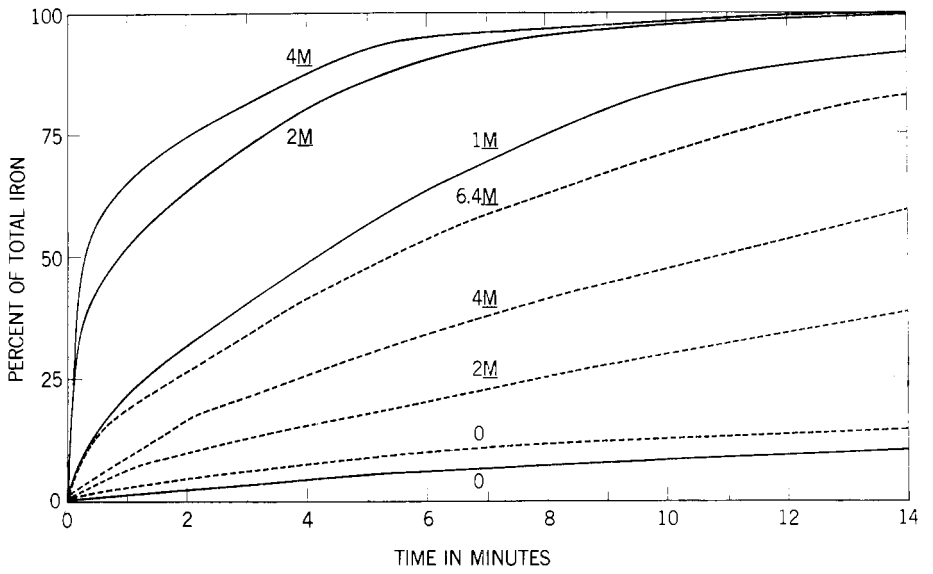

FIGURE 2: The reaction of ferredoxin with *o*-phenanthroline in urea and guanidine hydrochloride under aerobic conditions. Reaction mixtures contained 56 μg of *C. acidi-urici* ferredoxin equivalent to 77 mμatoms of iron, 0.1 M Tris-HCl (pH 7.4), 0.73 mM *o*-phenanthroline, and the indicated final concentration of urea (– – – –) or guanidine hydrochloride (———) in a final volume of 1.0 ml in 1.5-ml cuvets. The reaction was started by the addition of *o*-phenanthroline and the absorbancy change at 512 mμ was determined with a Gilford recording spectrophotometer.

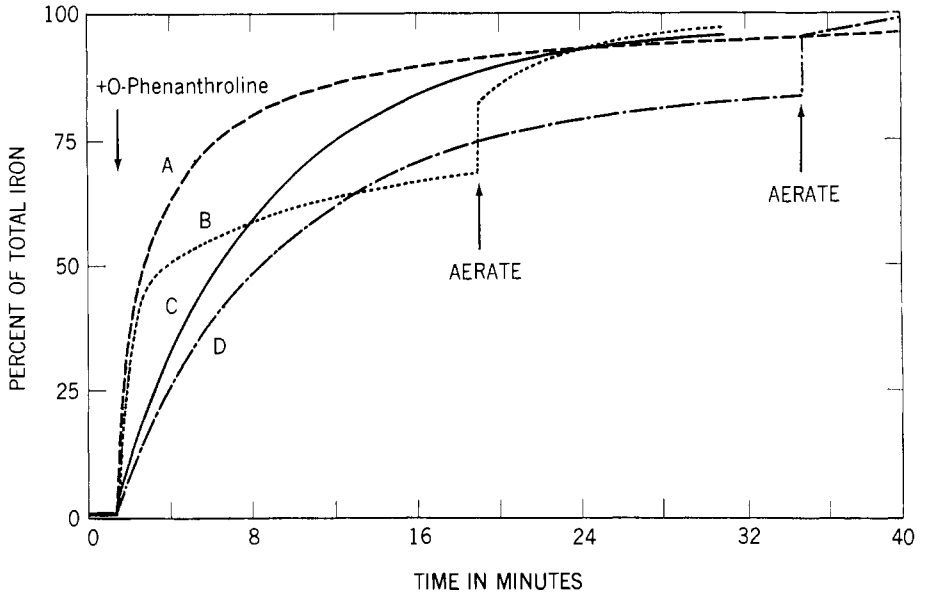

FIGURE 3: The reaction of ferredoxin with *o*-phenanthroline in urea and guanidine hydrochloride under anaerobic conditions. Reaction mixtures contained 43 μg of *C. acidi-urici* ferredoxin equivalent to 51 mμatoms of iron, 0.1 M Tris-HCl (pH 7.4), 0.73 mM *o*-phenanthroline, and 6.4 M urea or 4 M guanidine hydrochloride, in a final volume of 1.0 ml in 1.5-ml cuvets. Anaerobic samples were prepared as described in Figure 1. The reaction was started by the addition of *o*-phenanthroline and the absorbancy change at 512 mμ followed in the Cary Model 14 spectrophotometer. At the indicated times, the anaerobic samples were opened and vigorously aerated. (A) Guanidine hydrochloride (4 M), aerobic (– – – –); (B) guanidine hydrochloride (4 M), anaerobic (– – – –); (C) urea (6.4 M), aerobic (———); (D) urea (6.4 M), anaerobic (– · – ·).

of 4 and 2 M guanidine hydrochloride and the rates of the reaction in 1 M guanidine hydrochloride and 6.4 M urea were approximately equivalent. The reaction rate followed first-order kinetics in 6.4 M urea and 4 M guanidine hydrochloride with rate constants of 1.7 and 4.0 × 10^{-3} sec^{-1}, respectively. The difference in the reactivity of the iron toward *o*-phenanthroline in guanidine hydrochloride and urea may be due to the ionic nature of the former since ferredoxin is a highly acidic protein and may undergo structural changes more readily in the presence of the ionic denaturant, leading to the exposure of the iron atoms.

In the experiments described above, the reactions in 6.4 M urea and 4 M guanidine hydrochloride followed first-order kinetics and no kinetic discontinuities were seen over the period of the reaction. This implies that all the iron in the protein is kinetically equivalent under these conditions. Similar observations have been reported in preliminary studies (Gillard *et al.*, 1965). The rate of reduction of the ferric iron in the native protein must, therefore, be faster than the rate of reaction with the chelating agent.

The reaction of ferredoxin with *o*-phenanthroline under anaerobic conditions in 4 M guanidine hydrochloride proceeds to only 65% of completion (Figure 3B), whereas under aerobic conditions in 4 M guanidine hydrochloride (Figure 3A), 97% of the iron in the protein reacted with *o*-phenanthroline. The introduction of air to the anaerobic sample (Figure 3B, arrow) resulted in the complete reaction of the remaining iron. Similarly, under anaerobic conditions in 6.4 M urea, approximately 80% of the iron in the sample reacted with the chelating agent (Figure 3D), and the remaining 20% of the iron only became reactive after aeration (Figure 3D, arrow).

High concentrations of urea or guanidine hydrochloride allow the initial reaction of the chelating agent to occur with iron under either aerobic or anaerobic conditions. However, in the absence of oxygen, only 65–80% of the iron is reactive. This is equivalent to the reaction of approximately five to six atoms of iron per mole of ferredoxin. It is not clear whether the iron which is reactive under anaerobic conditions is composed of all ferrous iron of the protein and one to two atoms of ferric iron which have been reduced under these conditions, or whether the reactive iron is a mixture of ferric iron which has become reduced during the reaction and only a portion of the ferrous iron originally present. It is evident that some of the original ferric iron in the molecule must be reduced to the ferrous state under these reaction conditions.

In order to study the effect of oxygen on ferredoxin in more detail, the protein was incubated at room temperature in 4 M guanidine hydrochloride or 6.4 M urea under both anaerobic and aerobic conditions, and the spectra were recorded at different times. The results of this study are shown in Figure 4. Ferredoxin is

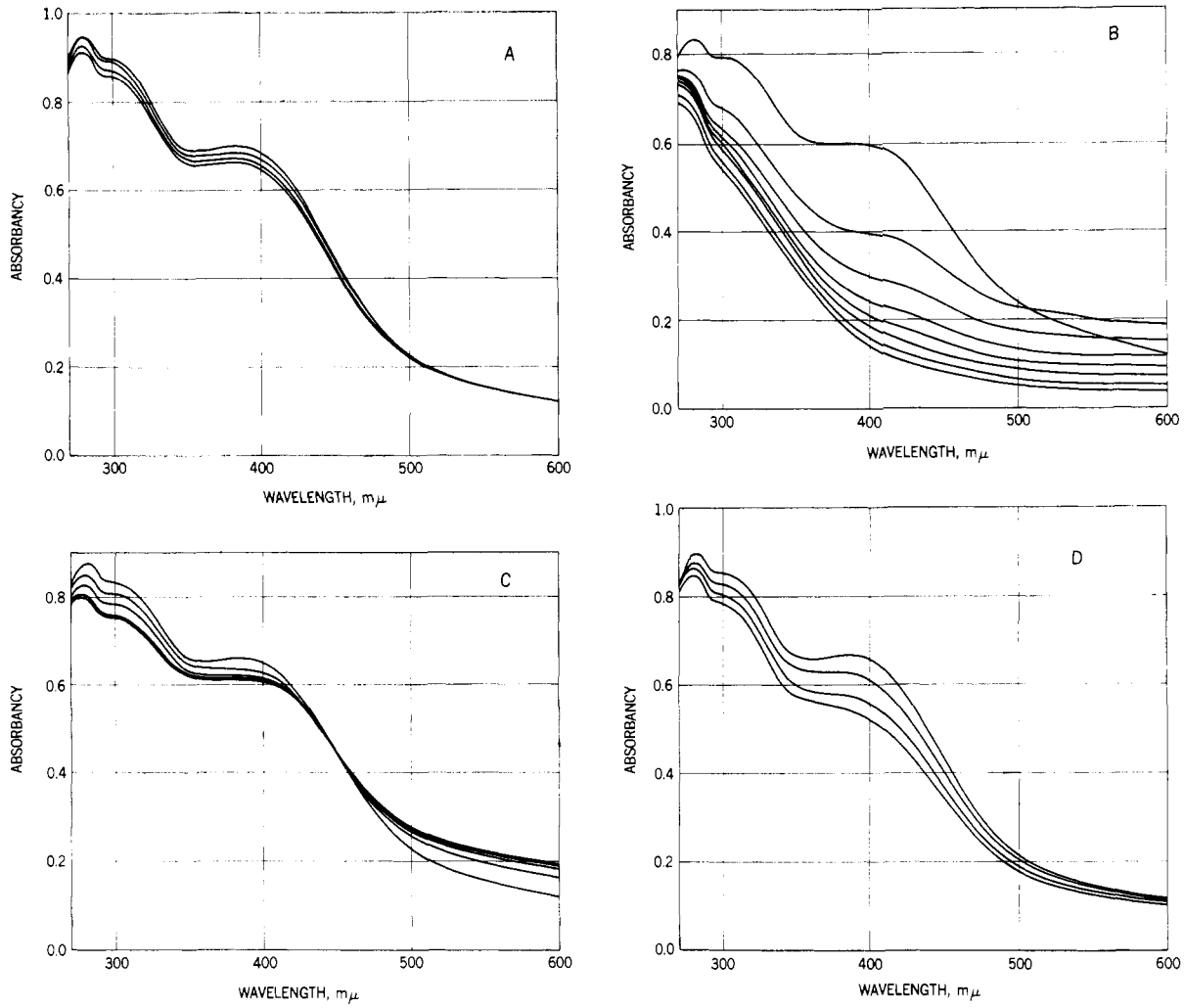

FIGURE 4: The stability of ferredoxin in urea and guanidine hydrochloride under aerobic and anaerobic conditions Reaction mixtures contained 128 μg of *C. acidi-urici* ferredoxin, 0.1 M Tris-HCl (pH 7.4), 4 M guanidine hydrochloride, or 6.4 M urea, where indicated, in a final volume of 1.0 ml in 1.5-ml cuvets. Anaerobic samples were prepared as described in Figure 1. The spectra of each sample were recorded at the indicated times after the addition of the protein. The starting spectrum, determined at 0 time, is the one with the highest absorption at 300 and 400 mμ in each case, and in the decreasing order as time progresses. (A) Guanidine hydrochloride (anaerobic conditions) spectra recorded at 0, 6, 20, and 75 min; (B) guanidine hydrochloride (aerobic conditions) spectra recorded at 0, 6, 12, 20, 30, 40, 60, and 75 min; (C) urea (anaerobic conditions) spectra recorded at 0, 6, 20, 45, and 60 min; (D) urea (aerobic conditions) spectra recorded at 0, 20, 45, and 60 min.

significantly more stable in 4 M guanidine hydrochloride and 6.4 M urea under anaerobic conditions than in the presence of oxygen. Over a 60-min period in 4 M guanidine hydrochloride under anaerobic conditions (Figure 4A), there was only a 6% decrease in absorption at 415 mμ, whereas under aerobic conditions (Figure 4B) there was approximately a 60% decrease. In 6.4 M urea under anaerobic conditions (Figure 4C), only a 10% decrease in absorption occurred at 415 mμ over the same time period as compared with a 26% decrease under aerobic conditions in this denaturant (Figure 4D).

The loss of the characteristic spectrum under these conditions suggests that this process results in a change in the iron and inorganic sulfide binding. The spectrum obtained, however, can be distinguished from that of the isolated apoprotein (Lovenberg *et al.*, 1963) by the relatively high absorption in the region below 400 mμ shown by the reaction mixture (Figure 4B). Whether this absorption is caused by the products formed from iron and sulfide and whether or not the products formed are associated with the protein have not been determined. It is not clear whether oxygen reacts directly with the iron atoms or with ligands of the iron. Experiments to be described in a later section, however, support the view that the inorganic sulfide in the molecule is the site of oxygen sensitivity. Iron and inorganic sulfide were released from the protein

under these conditions. This was demonstrated by isolating the protein obtained after treatment with 4 M guanidine hydrochloride under aerobic conditions at room temperature for 2 hr by passage over Sephadex G-25. The isolated protein, in contrast to the spectrum of the unfractionated reaction mixture (Figure 4B), was spectrally indistinguishable from the previously described apoferredoxins which contained no iron or sulfide (Malkin and Rabinowitz, 1966a).

It appears that the reaction of ferredoxin with *o*-phenanthroline under aerobic conditions is the combination of two reactions: the degradation of the protein in strong denaturants in the presence of oxygen and the reaction of the chelating agent with the metal. Thus, the interpretation of these experiments is complicated by the secondary effects of protein degradation. Under anaerobic conditions, however, the degradation of the protein is minimal, and the effect of the chelating agent is not complicated by these secondary reactions.

The Reaction of Clostridial Ferredoxin with Tiron. The reaction of ferredoxin with the ferric chelating agent, Tiron (sodium catecholdisulfonate), was followed by measuring the increase in extinction at 490 mμ. This reaction exhibited different characteristics from the reaction of ferredoxin with *o*-phenanthroline. Only a negligible reaction between native ferredoxin and Tiron could be detected under either anaerobic or aerobic conditions (Figure 5A) or by chemically and enzymatically reduced ferredoxin. In the presence of 6.4 M urea under either aerobic or anaerobic conditions, there is a slight reaction of ferredoxin with Tiron (Figure 5B), although under identical conditions, all of the iron in the protein reacted with *o*-phenanthroline. In the presence of 4 M guanidine hydrochloride under aerobic conditions, all the iron present, equivalent to 7–8 moles of iron, reacted with the chelating agent (Figure 5D). A first-order rate constant of 1.95×10^{-3} sec^{-1} was calculated for the reaction under these conditions.

Since all the iron in ferredoxin reacts with the ferric chelating agent under aerobic conditions, it was thought that oxygen was serving as the oxidant for the ferrous atoms present in the molecule. In the absence of oxygen, approximately half of the total iron present in ferredoxin reacts with the ferric chelating agent (Figure 5C). The hypothesis that oxygen can interact with the protein leading to further reaction of the remaining iron atoms was confirmed by aerating the reaction vessel after half the iron had reacted. The remaining iron then reacted, although not instantaneously, indicating that the iron was not fully exposed to the chelating agent (Figure 5C, arrow).

The observation that approximately half the iron in clostridial ferredoxin reacts with Tiron as ferric iron under anaerobic conditions is of interest since previous investigations (Sobel and Lovenberg, 1966; Palmer *et al.*, 1966) have supported the view that there are approximately equivalent amounts of ferric and ferrous iron in the native protein. In these previous experiments it was necessary to release the iron from the protein by treatment with a mercurial before determining the oxidation state, but in the experiments reported here, the valence of the iron has been determined under conditions in which the protein has been shown to be relatively stable. However, caution must still be exercised in interpreting these results since changes in the oxidation state of the iron could still have occurred in guanidine hydrochloride prior to the addition of the chelating agent even under anaerobic conditions and valence changes might occur during the course of the reaction with the chelating agent.

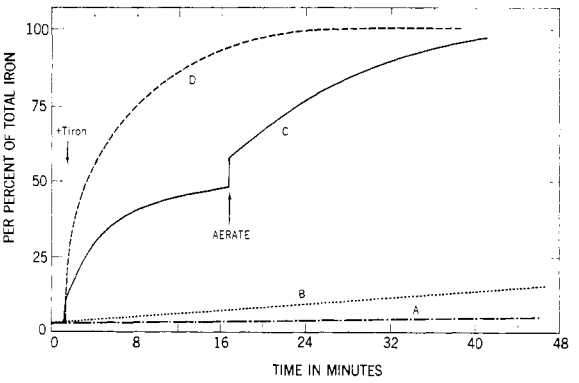

FIGURE 5: The reaction of ferredoxin with Tiron in urea and guanidine hydrochloride. Reaction mixtures contained 43 μg of *C. acidi-urici* ferredoxin equivalent to 51 mμatoms of iron, 0.1 M Tris-HCl (pH 7.4), 6.4 M urea, or 4 M guanidine hydrochloride where indicated, and 1 mM Tiron in a final volume of 1.0 ml in 1.5-ml cuvets. Anaerobic samples were prepared as described in Figure 1. The reaction was started by the addition of Tiron and the change in absorbancy at 490 mμ was followed in the Cary Model 14 spectrophotometer. At the indicated time, the anaerobic sample in guanidine hydrochloride was opened and vigorously aerated. All calculations were based on a millimolar extinction coefficient of 5.24 for the ferric–Tiron complex under these conditions. (A) Native ferredoxin (– · – ·); (B) ferredoxin in 6.4 M urea, anaerobic (– – – –); (C) ferredoxin in 4 M guanidine hydrochloride, anaerobic (———); (D) ferredoxin in 4 M guanidine hydrochloride, aerobic (- - - -).

The Reaction of the Inorganic Sulfide and Sulfhydryl Groups of Clostridial Ferredoxin with DTNB. Previous studies have shown that clostridial ferredoxin shows no spectral changes upon the addition of iodoacetate or *N*-ethylmaleimide, and that the cysteine residues of the protein could be alkylated with iodoacetate only after reduction with 2-mercaptoethanol in 8 M urea (Lovenberg *et al.*, 1963). In later experiments it was observed that the cysteine residues in native ferredoxin do not react with DTNB until the protein has been treated with sodium borohydride in 8 M urea (Malkin and Rabinowitz, 1966a). It was therefore concluded that the cysteine residues are blocked, presumably by the iron atoms present in the molecule.

Studies on the reactivity of the inorganic sulfide

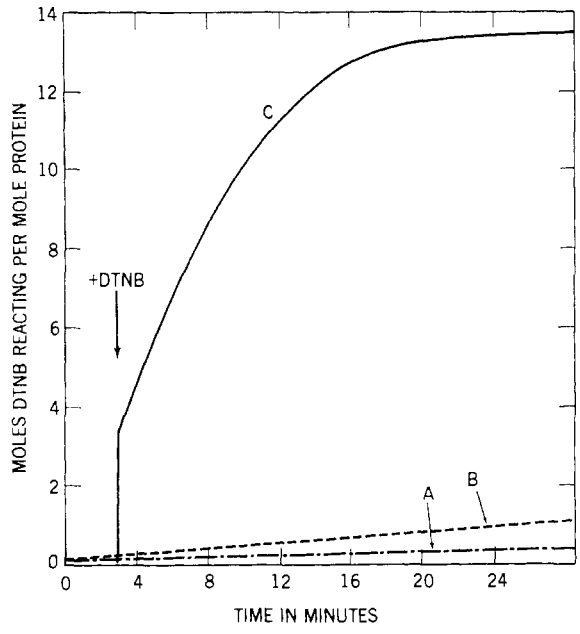

FIGURE 6: The reaction of ferredoxin with DTNB in urea and guanidine hydrochloride under anaerobic conditions. Reaction mixtures contained 24 μg of *C. acidi-urici* ferredoxin equivalent to 27 mμmoles of inorganic sulfide, 0.1 M Tris-HCl (pH 7.4), the indicated final concentration of urea (6.4 M) or guanidine hydrochloride (4 M), and 0.20 mM DTNB, in a final volume of 1.0 ml in 1.5-ml cuvets. Anaerobic samples were prepared as described in Figure 1. The reaction was started by the addition of DTNB and the absorbancy change at 412 mμ followed in the Cary Model 14 spectrophotometer. All calculations were based on a millimolar extinction coefficient of 13.6 for the reaction of DTNB with a solution of cysteine hydrochloride under these conditions. (A) Native ferredoxin (– · – ·); (B) ferredoxin in 6.4 M urea (– – – –); (C) ferredoxin in 4 M guanidine hydrochloride (———).

under these experimental conditions have not, however, been previously reported, nor has the effect of the reaction of the iron with various chelating agents on the sulfhydryl content of the protein been reported. Through the use of DTNB as a sensitive sulfhydryl reagent, the changes in the inorganic sulfide and sulfhydryl content of ferredoxin under various experimental conditions have been determined.

The effect of urea and guanidine hydrochloride on the reaction of ferredoxin with DTNB under anaerobic conditions is shown in Figure 6. Native ferredoxin does not react with DTNB during the 40-min period of the reaction (Figure 6A). It has been previously shown that the enzymatically reduced ferredoxin molecule does not react with an alkylating agent, indicating that there are no sulfhydryl groups exposed during the reduction of the protein (Sobel and Lovenberg, 1966). In the presence of 6.4 M urea, there was some reaction, as measured by the increase in extinction at 412 mμ (Figure 6B). Over a 40-min period, approximately 3 moles of DTNB reacted/mole of protein. The reaction in 4 M guanidine hydrochloride went to completion during this time period and followed first-order kinetics, with a rate constant of 1.59×10^{-3} sec^{-1} (Figure 6C). After the completion of the reaction in 4 M guanidine hydrochloride, 12.8 moles of DTNB had reacted/mole of ferredoxin. Control experiments indicated that 2 moles of DTNB react with 1 mole of sulfide, so that if only the inorganic sulfide had reacted under these conditions, one would expect 14.0 moles of DTNB to react/mole of protein. The stoichiometry of the reaction suggests that only the sulfide has reacted but it is also possible that a mixture of some cysteine residues and some sulfide has reacted. For example, the reaction of 8 cysteine residues and 2 moles of inorganic sulfide would give a total of 12 moles of DTNB reacting/mole of protein.

In order to determine whether the DTNB had reacted exclusively with the inorganic sulfide in the protein, the following experiment was performed. Ferredoxin was allowed to react with DTNB in 4 M guanidine hydrochloride under anaerobic conditions for 1 hr at room temperature. The extent of the reaction of the protein with DTNB was determined on a small aliquot of the reaction mixture. The remaining reaction mixture, which was connected to an all-glass bubbling train, was acidified to pH 3.0 and bubbled with nitrogen for 15 min to trap any evolved H_2S in a solution of zinc acetate. Control experiments indicated that sodium sulfide could be quantitatively diffused and trapped in the zinc acetate under these conditions, but that only 3% as much sulfide was recovered after reacting the sodium sulfide with DTNB prior to acidification. Thus, the product of the DTNB reaction with sulfide is stable under these conditions, and is not diffusible.

The results of an experiment with clostridial ferredoxin are shown in Table I. When ferredoxin was treated with DTNB in guanidine hydrochloride for 1 hr prior to acidification, only 0.3 mole of diffusible sulfide was recovered/mole of protein. Analysis of the reaction mixture before acidification showed that 14.0 moles of DTNB had reacted with the protein. The sample treated in an identical manner, in the absence of DTNB, led to the recovery of 7.0 moles of sulfide after acidification, an amount identical with a control sample which was added directly to the zinc acetate trap. This result confirms the conclusion that the DTNB reaction under anaerobic conditions in 4 M guanidine hydrochloride is due exclusively to the inorganic sulfide in ferredoxin and that there is no reaction of the cysteine residues under these conditions.

The reaction with DTNB was also studied under aerobic conditions and the results were similar to those obtained under anaerobic conditions. No reaction was detected with the native protein and a slight reaction occurred in 6.4 M urea. The reaction in 4 M guanidine hydrochloride was first order with a rate constant of 1.62×10^{-3} sec^{-1}, but only 10.5 moles of an expected 14.0 moles of DTNB reacted/mole of ferredoxin. This

TABLE I: The Effect of DTNB on the Release of Inorganic Sulfide from *C. acidi-urici* Ferredoxin.[a]

Sample	Moles of Sulfide Released/ Mole of Protein	Moles of DTNB Reacting/ Mole of Protein
Ferredoxin	7.0	
Ferredoxin + DTNB	0.3	14.0

[a] Reaction mixtures (in 13 × 100 mm tubes) contained 4 M guanidine hydrochloride, 0.10 M Tris-HCl (pH 7.4), 0.34 mg of *C. acidi-urici* ferredoxin, and 4 mM DTNB, where present, in a final volume of 1.0 ml. The reaction vessel was attached to a trapping tube, containing 1 ml of 1% zinc acetate, in an all-glass bubbling train. The reaction vessel was flushed with prepurified nitrogen for 1 hr at room temperature prior to the addition of the protein and DTNB. The reaction was started by the addition of the protein and allowed to proceed for 1 hr without any nitrogen bubbling. Aliquots were widthdrawn from the tube containing DTNB and the absorbancy at 412 mμ was determined in order to determine the extent of the protein sulfhydryl reaction. Glacial acetic acid (0.050 ml) was then added to each reaction vessel to bring the pH to approximately 3.0, and nitrogen was bubbled through the reaction mixtures for 15 min. Control experiments showed a 90% recovery of sodium sulfide under these conditions in the absence of DTNB and only a 3% recovery in the presence of DTNB. The traps were vigorously stirred to resuspend any precipitated sulfide and aliquots were assayed for inorganic sulfide as described under Methods. Duplicate determinations were carried out for each trap. A control, which contained no ferredoxin, but to which ferredoxin (0.34 mg) had been added directly to the trap, had an inorganic sulfide content of 7.4 moles/mole of protein.

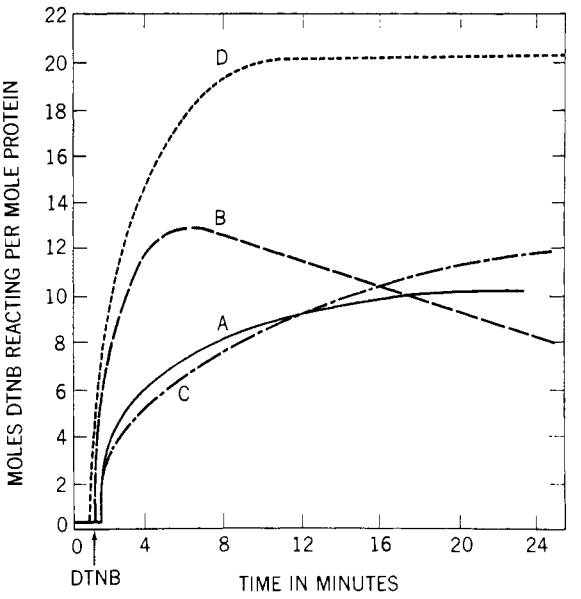

FIGURE 7: The effect of EDTA on the reaction of DTNB with ferredoxin in 4 M guanidine hydrochloride. Reaction mixtures contained 16 μg of *C. acidi-urici* ferredoxin equivalent to 18 mμmoles of inorganic sulfide, 0.1 M Tris-HCl (pH 7.4), 4 M guanidine hydrochloride, 0.20 mM DTNB, and 1 mM EDTA, where present, in a final volume of 1.0 ml in 1.5-ml cuvets. Anaerobic samples were prepared as described in Figure 1. The reaction was started by the addition of DTNB and the absorbancy change at 412 mμ followed in the Cary Model 14 spectrophotometer. Aerobic, no EDTA (———); (B) aerobic, with EDTA (– – –); (C) anaerobic, no EDTA (– · – ·); (D) anaerobic, with EDTA (- - - -).

apparent incomplete reaction may be due to oxidation of some of the sulfide under aerobic conditions so that it is no longer in a form which reacts with DTNB.

A possible explanation for the lack of reactivity of the cysteine residues with DTNB in 4 M guanidine hydrochloride is that these sulfhydryl groups were oxidized in the presence of the ferric iron of the protein to form disulfides. This hypothesis was tested by allowing ferredoxin to react with DTNB in 4 M guanidine hydrochloride in the presence of EDTA to prevent possible metal catalysis of sulfhydryl oxidation.

Under aerobic conditions in the absence of EDTA, it was found that 10.5 moles of DTNB react/mole of protein (Figure 7A). The protein sample contained 6.8 moles of inorganic sulfide/mole so that a reaction of all the sulfide would give a final reaction of 13.6 moles of DTNB/mole of protein. In the presence of 1 μmole of EDTA (Figure 7B), the rate of reaction is faster than in its absence, and at the point of maximum reaction, 13.2 moles of DTNB had reacted. However, the absorption generated during the reaction was not stable and faded rapidly. A similar observation on the fading of the color in the DTNB reaction in concentrated solutions of guanidine hydrochloride was reported by Kaplan and Flavin (1966), and these authors suggested that oxygen is involved since the color could be stabilized by the addition of ascorbate.

Under anaerobic conditions, the reaction shows different characteristics. In the absence of EDTA, approximately 13 moles of DTNB reacted/mole of protein, indicating a complete reaction of the inorganic sulfide (Figure 7C). This absorption did not decrease with time under these conditions in agreement with the hypothesis that oxygen is involved in the loss of absorption. In the presence of EDTA, 21.1 moles of DTNB react (Figure 7D). This can be accounted for on the basis of the complete reaction of the cysteine and inorganic sulfide content of the ferredoxin.

Since the reaction of DTNB with all of both the sulfide and cysteine residues of ferredoxin occurs

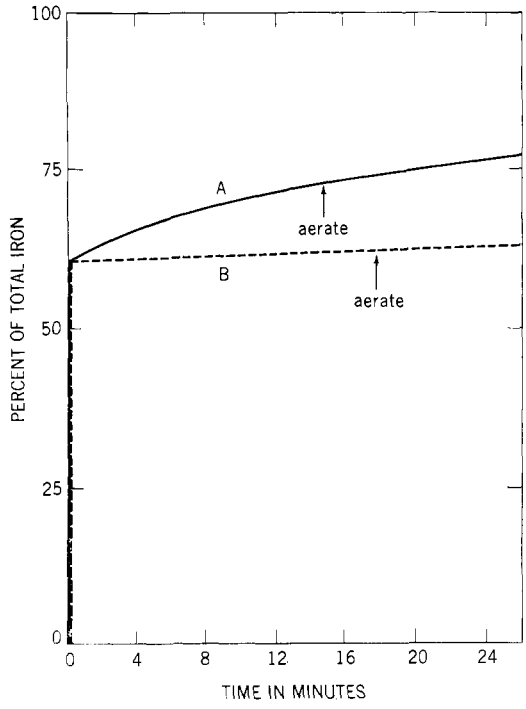

FIGURE 8: The reaction of ferredoxin with iron chelating agents after the reaction with DTNB. Reaction mixtures contained, in 1.5-ml cuvets, 36 (A) or 109 μg (B) of *C. acidi-urici* ferredoxin, 0.1 M Tris-HCl (pH 7.4), 4 M guanidine hydrochloride, 0.20 mM DTNB, and either 0.40 mM sulfonated bathophenanthroline (A) or 1.0 mM Tiron (B), in a final volume of 1.0 ml. Anaerobic samples were prepared as described in Figure 1 except that all solutions were flushed with hydrogen for 30 min. The reaction was started by the addition of DTNB and the absorbancy change at 412 mμ followed in the Cary Model 14 spectrophotometer for 40 min in the case of reaction A. In A, 14.2 moles of sulfhydryl reacted/mole of protein over this time period. It was not possible to follow the kinetics of the DTNB reaction in part B because of the greater amount of protein used and the reaction mixture was incubated for 1 hr at room temperature. Tiron or sulfonated bathophenanthroline were added at the conclusion of the DTNB reaction and the reaction followed at 540 and 535 mμ, respectively. The cuvets were vigorously aerated at the indicated times. Only the time course of the reaction with the respective iron chelating agent is shown in the figure.

only under anaerobic conditions in the presence of EDTA, it can be concluded that both the iron atoms and oxygen are involved in the oxidation of the cysteine sulfhydryl groups to the disulfides. These experiments do not, however, allow the determination of any change in the valence state of iron during the sulfhydryl oxidation since EDTA gives no colored complexes with either ion.

In order to determine the oxidation state of the iron during the DTNB reaction with ferredoxin, the protein was incubated with DTNB under anaerobic conditions in 4 M guanidine hydrochloride in the presence of either a ferric or ferrous chelating agent. The effect of the reaction of DTNB with ferredoxin on the reactivity of the iron in the protein was determined by adding the iron chelating agent after the completion of the reaction of DTNB with the inorganic sulfide of the protein.

The experiments with DTNB and either the ferric chelating agent, Tiron, or the ferrous chelating agent, sulfonated bathophenanthroline, are complicated by the fact that both the DTNB product and the iron chelate complex absorb at 412 mμ. In addition, it was found that the DTNB reaction color contributed significant absorption at 490 mμ, the absorption maximum for the ferric–Tiron complex. The analysis of the iron complexes in the presence of DTNB is described in the Methods. However, the DTNB color at 535 mμ, the absorption maximum for the ferrous–sulfonated bathophenanthroline complex, was negligible and no correction had to be made at this wavelength.

When DTNB and Tiron were added to ferredoxin in 4 M guanidine hydrochloride under anaerobic conditions and the absorbancy change at 412 or 540 mμ followed, both the iron and sulfhydryl reactions are complete in less than 10 min. A similar result was obtained if DTNB and sulfonated bathophenanthroline were added together at the start of the reaction and the absorbancy change followed at either 412 or 535 mμ.

The quantitative results of an experiment in which ferredoxin was treated with DTNB and an iron chelating agent are shown in Table II. When ferredoxin is treated with DTNB in the presence of the ferrous chelating agent sulfonated bathophenanthroline, approximately 15 moles of DTNB react/mole of protein. This is an indication that only the inorganic sulfide in the protein reacted with DTNB under these conditions. If the extinction at 535 mμ is measured at the completion of the reaction and used to estimate the amount of iron present at the conclusion of the reaction as the ferrous–bathophenanthroline complex, it is found that 7.4 atoms of iron/mole of protein have reacted as ferrous iron. Since the sample contains 7.2 atoms of iron/mole of protein by iron analysis as described in Methods, all the iron has reacted with the ferrous chelating agent. Since only inorganic sulfide reacts with DTNB under these conditions, it may be concluded that the ferric iron orginally present in the protein has been reduced to the ferrous state at the expense of the oxidation of the cysteine residues to disulfides so that the latter can no longer react with DTNB.

When ferredoxin reacts with DTNB in the presence of the ferric chelating agent (Tiron), approximately 25 moles of DTNB react/mole of protein and 7.6 atoms of iron appear as the ferric–Tiron complex (Table II). In the presence of the ferric chelating agent and DTNB, the iron of ferredoxin appears entirely as the ferric–Tiron complex and all the sulfur in the

TABLE II: The Reaction of *C. acidi-urici* Ferredoxin with DTNB in the Presence of Iron Reagents.[a]

Sample	Moles of DTNB Reacting/ Mole of Protein	Atoms of Iron Reacting Mole of Protein
Ferredoxin + DTNB + sulfonated bathophenanthroline	14.5	7.5
Ferredoxin + DTNB + Tiron	24.6	7.6

[a] Reaction mixtures contained in 1.5-ml cuvets 34 μg of *C. acidic-urici* ferredoxin containing 40 mμmoles of inorganic sulfide and 41 mμmoles of iron, 0.1 M Tris-HCl (pH 7.5), 4 M guanidine hydrochloride, 0.20 mM DTNB, and 1 mM Tiron or 0.50 mM sulfonated bathophenanthroline, where present, in a final volume of 1.0 ml. Anaerobic samples were prepared as described in Figure 1 except that all solutions were flushed with hydrogen gas for 30 min at room temperature. The reaction was started by the addition of the iron reagent and DTNB. The absorbancy change at 412 mμ, 540 mμ for the ferric solution, or 535 mμ for the ferrous complex was followed in the Cary Model 14 spectrophotometer. Duplicate determinations were performed for each sample, the first time following the sulfhydryl reaction and the second time following the iron reaction. All calculations were carried out as described in the text.

protein is in a form which reacts with DTNB. This finding that both the inorganic sulfide and cysteine sulfhydryl groups of ferredoxin react with DTNB under these conditions is consistent with the result obtained when ferredoxin was allowed to react with DTNB in the presence of EDTA since the reduction of the iron at the expense of the cysteine residues cannot occur when the ferric iron is chelated. The observation that all the iron in the sample now reacts as ferric iron is unusual, however, since it was not anticipated that the ferrous iron of the native protein would be oxidized to ferric iron under these reaction conditions.

Control experiments carried out in the absence of ferredoxin indicated that DTNB itself can oxidize ferrous iron in the presence of Tiron. The quantitative conversion of ferrous iron to ferric iron could be shown but the nature of the reaction was not clear since a stoichiometric production of the second reaction product, the thiophenol formed from DTNB, could not be demonstrated. Since the oxidation of ferrous iron by DTNB occurs readily in the presence of Tiron, the extent of the protein sulfhydryl reaction in the presence of Tiron and DTNB will be higher than that expected because of the production of the thiophenol product by the reaction which occurs in the absence of protein. This suggests that the amount of DTNB reacting with ferredoxin in the presence of Tiron reported in Table II is higher than the amount caused by the protein sulfhydryl groups alone, so that this result cannot be taken as conclusive evidence for the reaction of all the sulfur in the protein as sulfhydryls under these conditions.

The effect of the reaction of the inorganic sulfide with DTNB on the reactivity of the iron in ferredoxin was determined by adding DTNB to ferredoxin under anaerobic conditions in 4 M guanidine hydrochloride and allowing the reaction to go to completion; under these conditions only the inorganic sulfide will react with DTNB. At the end of the reaction, either a ferric or ferrous chelating agent was added and the reaction of the iron with the chelating agent was observed.

When the ferrous chelating agent sulfonated bathophenanthroline was added, there was an instantaneous reaction of approximately 60% of the total iron in the sample, as shown in Figure 8A. This rapid reaction was followed by a much slower one, during which another portion of the iron reacted. In addition, at the time indicated in the figure, the sample was aerated, but aeration did not affect the rate of reaction of the remaining iron under these conditions.

When a similar reaction was carried out with the addition of Tiron at the completion of the DTNB reaction, the result was similar to that described with the ferrous chelating agent. As shown in Figure 8B, there was an initial instantaneous reaction of 60% of the total iron with the ferric chelating agent, followed by a much slower reaction, and aeration did not affect the latter rate. This initial reaction may be due to the reaction of both ferric iron in the native protein and the reaction of some ferric iron produced through the reaction of DTNB with available ferrous iron in the protein, but the significant finding is that under these conditions the reaction does not go to completion and all the iron does not react as ferric iron, in contrast to the results found when both Tiron and DTNB are added together at the start of the reaction.

Discussion

Optical rotatory dispersion and circular dichroism spectra support the view that changes in the environment of the chromophoric group in bacterial ferredoxin occur upon reduction of the protein (Gillard *et al.*, 1965; Atherton *et al.*, 1966). The chemical reactivity of oxidized and reduced clostridial ferredoxin toward the ferrous chelating agent *o*-phenanthroline is also consistent with this concept since the removal of iron from the reduced protein is much more difficult and shows different kinetic characteristics than with the native material, even though in the latter case, the reaction of *C. acidi-urici* ferredoxin with the chelating agent is not instantaneous and is relatively slow.

The results of Gillard *et al.* (1965, 1966) on the reactivity of native *C. acidi-urici* ferredoxin do not agree with some of the observations reported here.

These workers claim that they could detect no formation of the ferrous–*o*-phenanthroline complex up to 30 min after the addition of the chelating agent (Gillard *et al.*, 1966). In the presence of 8 M urea, however, the iron was reactive and the rate of the formation of the chelate complex followed first-order kinetics. Although the latter observation agrees with the results shown in Figure 2 in which both urea and guanidine hydrochloride markedly affect the reactivity of the iron in ferredoxin, it is difficult to reconcile the finding that no iron was removed from the native protein with the findings reported here. The same experiment has been repeated with different samples of ferredoxin and all reacted under identical conditions at essentially the same rate in the presence of *o*-phenanthroline. In contrast to the observation with *C. acidi-urici* ferredoxin, Gillard *et al.* (1966) have found that the iron is removed from the native ferredoxins from *C. pasteurianum* and *Peptostreptococcus elsdenii* in the presence of *o*-phenanthroline.

The studies on the reactivity of the iron in the presence of either urea or guanidine hydrochloride indicate that the iron environment is not readily accessible to chelating agents and is consistent with the structure in which the iron atoms are not fully exposed at the surface of the molecule but are "buried," and not capable of direct reaction with the added chelating agent. In an early study with succinic dehydrogenase, Massey (1958) also found that the nonheme iron present in the protein is relatively inaccessible for reaction with iron chelating agents. This observation implies that bacterial ferredoxin contains exposed and unexposed sites and that the protein has a conformation in which the iron atoms are unexposed. This should not, however, be taken to imply that ferredoxin is a protein containing significant regions of helicity since data from the amino acid sequence (Tanaka *et al.*, 1966) and optical rotatory dispersion spectrum in the ultraviolet region (Lovenberg, 1966) have suggested that bacterial ferredoxin contains few regions of helicity.

In addition to changes in reactivity upon addition of the denaturants (urea or guanidine hydrochloride), the most striking effects in the studies on the iron reactivity of ferredoxin are the demonstrations of differential reactivity of the iron atoms. The experiments carried out under anaerobic conditions in the presence of denaturants demonstrate that all the iron in the protein is not reactive toward either a ferric or ferrous chelating agent, but that the reactivity of the iron increases in the presence of oxygen and the reactions only go to completion in the presence of oxygen. It was found that the protein undergoes rapid degradation in denaturants in the presence of oxygen but that relatively little degradation occurs under the same conditions in the absence of oxygen. The degradation of clostridial ferredoxin can be followed spectrally by the decrease in absorption at 390 mμ, and these changes are indicative of the loss of iron and sulfide from the protein.

Experiments on the oxygen sensitivity of alfalfa ferredoxin by Keresztes-Nagy and Margoliash (1966) have also been reported, and this protein appears to be much more sensitive than clostridial ferredoxin since deterioration readily occurs in the presence of oxygen even in the absence of any denaturant. However, it is significant to note that these workers found that alfalfa ferredoxin could be protected indefinitely by storage under nitrogen gas. Thus, it may be that the slow deterioration of clostridial ferredoxin which occurs with the ferredoxin from *C. pasteurianum* and the rapid deterioration which occurs with samples from such species as *C. cylindrosporum* (Lovenberg *et al.*, 1963) could be prevented by storage of these ferredoxins under anaerobic conditions.

The conditions under which the inorganic sulfide and sulfhydryl groups of ferredoxin react with DTNB were also studied. In contrast to the effect of urea on the reactivity of iron, this reagent had relatively little effect in enhancing the reaction of the sulfide with DTNB, but a reaction could be readily demonstrated in the presence of 4 M guanidine hydrochloride. The reaction of the cysteine sulfhydryl groups with DTNB could only be demonstrated under anaerobic conditions in the presence of 4 M guanidine hydrochloride and EDTA. The requirement for EDTA suggests that the iron in ferredoxin is involved in the oxidation of the cysteine residues to the disulfides under conditions where no precautions are taken to chelate the ferric iron. Direct evidence for the participation of the cysteine residues in ferric iron reduction was demonstrated in experiments in which DTNB and a ferrous chelating agent were added together to ferredoxin in 4 M guanidine hydrochloride under anaerobic conditions.

The sulfhydryl groups of ferredoxin are capable of reducing the ferric iron in the protein to the ferrous state and the rate of reaction of the ferrous iron with an added chelating agent is slower than the rate of reduction. This is demonstrated by the kinetics of the reaction of ferredoxin with *o*-phenanthroline in urea or guanidine hydrochloride. However, from this first experiment it is not possible to differentiate between the two sulfhydryl reducing groups in the protein, inorganic sulfide, and cysteine residues. Under conditions in which the inorganic sulfide of ferredoxin reacts with DTNB, the cysteine residues are not found as free sulfhydryls and all the iron in the protein sample appears in the ferrous state. This suggests that only the cysteine residues are involved in the reduction of the ferric iron and that the inorganic sulfide in the molecule does not participate in this reaction.

If the inorganic sulfide of ferredoxin is allowed to react completely with DTNB in 4 M guanidine hydrochloride under anaerobic conditions, and an iron chelating agent is then added, the reactivity of the iron shows different characteristics from that of the iron in protein which is exposed only to 4 M guanidine hydrochloride. Approximately 60% of the iron reacts immediately upon the addition of either a ferrous or ferric chelating agent, which strongly suggests that this iron has been released from the protein or has been fully exposed during the anaerobic reaction of the sulfide with DTNB. The reaction of the remaining iron, however, is slower

than the reaction of the iron in the protein in 4 M guanidine hydrochloride alone and is not affected by the presence of oxygen. This result supports a model in which the slow-reacting iron is still retained on the protein and is relatively inaccessible to the chelating agents due to conformational changes which have occurred during the DTNB reaction. It is also implied that the oxygen-labile groups have been lost during this first reaction since the slow reaction of the iron cannot be stimulated by oxygen.

Studies on alfalfa ferredoxin (Keresztes-Nagy and Margoliash, 1966) have also shown that the removal of the inorganic sulfide from the protein by extensive dialysis or heating led to the formation of a protein derivative in which the iron was bound more tightly than in the native protein. In addition, deterioration was always accompanied by the loss of sulfide, and it was suggested in this work that the aerobically sensitive site in the protein which led to deterioration was the sulfide.

This finding is totally consistent with the observations reported here. Although all the iron of the ferredoxin reacts with *o*-phenanthroline in the presence of air and a denaturant, after it has been treated with DTNB, 60% of the iron reacts instantaneously with *o*-phenanthroline, but the remaining 40% is now unreactive with the iron chelating agent under anaerobic as well as aerobic conditions in 4 M guanidine hydrochloride.

It is not clear from these experiments whether the iron remaining on the protein is still bonded to its original ligand, presumably the cysteine residues, or has become chelated to some other groups, such as the carboxyl groups of the acidic residues. It is likely that the bonding is not nonspecific because of the high affinity of the protein for the iron in the presence of the added chelating agents. However, the effect of the protein structure in protecting the iron from reaction cannot be directly assessed and only isolation and characterization of this protein derivative can resolve this question.

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