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Further Observations on the Chemical Nature of Rubredoxin from *Clostridium pasteurianum**

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With the Technical Assistance of Eleanor C. Bruckwick

ABSTRACT: The nature of the iron binding of rubredoxin undoubtedly accounts for the redox properties of the protein. The half-cysteine residues in the protein appear to form four of the ligands of the iron. This conclusion was derived from measuring the reactivity of the protein toward sulfhydryl reagents (iodoacetate and mercurials) and the finding that 2-mercaptoethanol was required for an ^{59}Fe exchange reaction and for reconstitution of the apoprotein. It was also observed that rubredoxin could undergo its complete redox cycle in the crystalline state without apparent damage to the crystal. Finally, an improved technique for the isolation of rubredoxin from *Clostridium pasteurianum* is described.

Rubredoxin is a nonheme iron protein which has been found to be present in many clostridial species (Lovenberg and Sobel, 1965; Stadtman, 1965; Mayhew and Peel, 1966; LeGall and Dragoni, 1966). While no unique physiological role has been established for this clostridial protein, a protein containing a similar iron chromophore has been isolated and shown to participate in hydrocarbon oxidation in *Pseudomonas oleovorans* (Peterson and Coon, 1968). Previous studies (Lovenberg and Sobel, 1965) in this laboratory have shown the clostridial protein to have a molecular weight of about 6000 and to contain one atom of iron. Rubredoxin has absorption maxima at 490, 380, and 280 mμ, shows optical activity (Lovenberg, 1966), and undergoes reversible one-electron oxidation and reduction with a redox potential of about −0.057 V (Lovenberg and Sobel, 1965). Rubredoxin exhibits an electron spin resonance signal at $g = 4.3$ in the oxidized state but no observable signal in the reduced state (Lovenberg, 1966). Recently the complete amino acid sequences of rubredoxin from *Micrococcus aerogenes* and *Peptostreptococcus elsdenii* have been reported (Bachmayer *et al.*, 1967a, 1968a).

It has been suggested (Lovenberg, 1966; Bachmayer *et al.*, 1967a,b) that the cysteine residues may participate in the iron binding properties of this protein. The current study was undertaken to further clarify the role of the cysteines and to examine possible molecular changes which occur during the oxidation–reduction cycle of the protein. In addition an improved method for isolation of the protein is reported.

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Materials

[1-^{14}C]Iodoacetic acid (1.4 μCi/μmole), $^{59}FeCl_3$ (2.9 mCi/μmole), and omnifluor scintillator solution were obtained from New England Nuclear Corp. The iodoacetic acid (100 μCi) was mixed with 1.5 g of unlabeled material dissolved in a minimal volume of chloroform and recrystallized. The white crystalline material had a specific activity of 2.22×10^4 cpm/μmole under standard counting conditions. Other reagents were the best grades available from commercial sources. *Clostridium pasteurianum* was grown on a medium containing $(NH_4)_2SO_4$ as its sole nitrogen source as described previously (Lovenberg *et al.*, 1963).

Rubredoxin was isolated from frozen cells (Lovenberg and Sobel, 1965) through the second DEAE-cellulose step. In this step the 90% saturated $(NH_4)_2SO_4$ solution is applied to a DEAE-cellulose column and the mixture of ferredoxin and rubredoxin is adsorbed to the ion-exchange resin. By use of a decreasing gradient of $(NH_4)_2SO_4$ concentration it is possible to obtain sequential elution of these two proteins. As seen in Figure 1 when approximately 1 mg each of the two highly purified proteins was applied as a mixture to a 1.0×10 cm DEAE-cellulose in a 90% saturated $(NH_4)_2SO_4$ solution and a linear gradient of 100% saturated $(NH_4)_2SO_4$ to water applied, rubredoxin (peak A) was eluted starting at about 45% saturation and ferredoxin (peak B) at about 35% saturation. Following this step the rubredoxin was concentrated by increasing the $(NH_4)_2SO_4$ concentration to about 80% and readsorbing on a small DEAE-cellulose column. The rubredoxin was eluted from the column with 0.8 M Cl^- buffer (0.65 M NaCl and 0.15 M Tris-HCl, pH 7.3). The rubredoxin was next de-

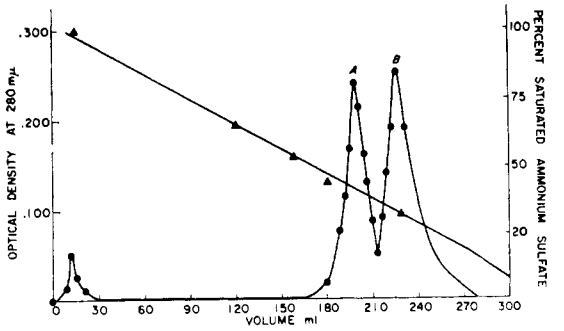


FIGURE 1: The separation of rubredoxin and ferredoxin by a negative $(NH_4)_2SO_4$ gradient on a DEAE-cellulose column. Approximately 1 mg each of pure ferredoxin and rubredoxin were dissolved in 50 ml of water. Solid $(NH_4)_2SO_4$ was added to bring the solution to 90% saturation with respect to $(NH_4)_2SO_4$ and the sample applied to a 1.2 × 10 cm DEAE-cellulose column. The proteins were eluted with a linear gradient from a Phoenix Varigard gradient mixer. The first chamber contained about 150 g of saturated $(NH_4)_2SO_4$ solution and the second chamber contained about 150 g of water. Fractions (4 ml) were collected and optical density at 280 mμ (●—●) and $(NH_4)_2SO_4$ concentration (▲—▲) were determined. The latter measurements were done by conductivity.

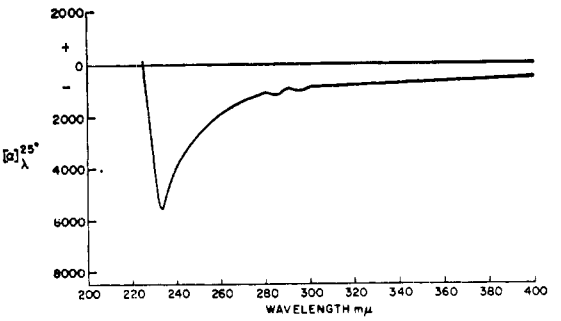


FIGURE 2: The optical rotatory dispersion spectrum of aporubredoxin. The spectrum was obtained using a solution containing 0.044 mM aporubredoxin and 0.05 M Tris-HCl (pH 7.3) in a cell with a path length of 1 cm at room temperature.

salted on G-25 Sephadex and the protein was lyophilized. The rubredoxin was dissolved in water (2–5 mg/ml) and solid $(NH_4)_2SO_4$ was added until a slight turbidity began to appear. Upon standing overnight large (1 mm long) crystals usually appeared in the mother liquor. During the course of isolation the purity of rubredoxin was ascertained by an absorbancy ratio at $A_{280\ m\mu}/A_{490\ m\mu}$. This ratio was found to be 2.4 or slightly lower for the crystalline protein (Lovenberg and Sobel, 1965). Rubredoxin appeared to be stable after isolation with little or no decomposition being noted after storage for 1 month at 4° in neutral solutions.

Aporubredoxin was prepared from rubredoxin by precipitation of the protein with 8% trichloroacetic acid. Decomposition of iron chromophore was accelerated by warming the suspension to 50–60°. The precipitate was removed by centrifugation and dissolved in a warm solution of ethanol (70%) containing 0.05% 1,10-phenanthroline. The protein was then reprecipitated by cooling to 0° for 15 min. The precipitate was washed two times with ethanol and dried. Upon solution in dilute Tris buffer the material was colorless and showed a single protein absorbancy maximum at 280 mμ (ϵ_{280} 18.5×10^{-3} M^{-1} cm^{-1}). An optical rotatory dispersion spectrum (Figure 2) was that of a typical protein. In fact the trough at 233 mμ was similar to that in native rubredoxin; *vide infra*. The number of free sulfhydryl groups was also determined to be 2.7 in a single preparation of aporubredoxin by a *p*-mercuribenzoate titration. This was done according to the procedure of Boyer (1954) using various amounts of aporubredoxin in 0.1 M potassium phosphate buffer (pH 6.8) and 10^{-5} M *p*-mercuribenzoate. Since this is appreciably less than the four found in native rubredoxin (Lovenberg, 1966) it is apparent that some oxidation of the sulfhydryl groups is taking place during the isolation of aporubredoxin.

Hydrogenase. Frozen cells of *C. pasteurianum* were suspended in about two volumes of water and sonically disrupted for 5 min in a Biosonik 20-kc sonic oscillator at 0°. The resulting suspension was centrifuged at 20,000*g* for 10 min. The ferredoxin and rubredoxin were removed from the supernatant fraction by passage of the extract through a small DEAE-cellulose column (Buchanan *et al.*, 1963). The final extract contained about 5 mg of protein/ml.

Methods

Protein Hydrolysis. Samples, usually about 0.2 mg of protein, were dissolved in 0.5 ml of 6 N HCl in hydrolysis tubes which then were repeatedly evacuated and flushed with nitrogen for 30 min. The tubes were then evacuated, sealed, and placed in a constant-temperature oil bath at 110° for varying lengths of time.

Tryptophan Determination. A modification of the standard fluorescence analysis (Udenfriend, 1962) was used. Samples of rubredoxin and tryptophan standards were hydrolyzed in 1 ml of 5 N NaOH for 20 hr in an autoclave. The solutions were neutralized with 0.6 ml of 10 N H_2SO_4 and diluted to 10 ml with water; 1 ml of this solution was added to 4 ml of 1 M Na_2CO_3 and the fluorescence was determined in an Aminco Bowman spectrophotofluorometer (excitation 300 mμ and fluorescence 360 mμ). The amounts of tryptophan in the protein hydrolysates were then determined by comparison with the internal standards carried through the entire procedure.

Rubredoxin Derivatives. Cysteic acid derivative of rubredoxin was prepared by the method of Hirs (1956). Carboxymethylrubredoxin was prepared by a slight modification of the method of Canfield and Anfinsen (1963). Aporubredoxin (4 mg) was dissolved in 1 ml of 1.0 M Tris buffer (pH 8.6) containing 0.5 M 2-mercaptoethanol and 8 M urea. The tube containing the mixture was stoppered and the solution was bubbled thoroughly with nitrogen and allowed to remain under anaerobic conditions for at least 4 hr. The tube was then opened and solid iodoacetamide was added until the solution gave a negative nitroprusside test. After 15 min the solution was diluted to 3 ml and the protein was isolated on a Sephadex G-25 column.

Iron-Exchange Studies. The exchange of exogenous radioactive iron with the iron of rubredoxin was studied under various conditions during incubation at 37°. The incubation mixture contained 0.1 M Tris buffer, rubredoxin, 0.5 μmole of iron salts (3×10^6 cpm/μmole), and 20 μmoles of 2-mercaptoethanol in a total volume of 0.2 ml. At the conclusion of the incubation 0.75 μmole of 1,10-phenanthroline was added and the mixture was chromatographed on a 0.5 × 20 cm G-25 Sephadex column. The rubredoxin-containing fraction was examined by counting 0.1 ml in omnifluor solution and determining the rubredoxin concentration (optical density at 490 mμ) in the remaining fraction. Results are expressed as per cent equilibration = (specific activity of rubredoxin/specific activity of total iron in the system) × 100.

Iron Determination. Iron was determined by the modification of the procedure of Harvey *et al.* (1955) reported previously by Lovenberg *et al.* (1963) and by the bathophenanthroline method of Peterson (1953).

Instrumentation. Optical rotatory dispersion spectra were recorded in a Cary Model 60 spectropolarimeter and absorption spectra were observed in a Cary Model 15 spectrophotometer. Radioactivity measurements were done in an omnifluor solution using a Packard 3375 liquid scintillation spectrometer. Amino acid analyses were done with a Beckman 120C amino acid analyzer equipped for high-sensitivity analysis.

Results

Oxidation and Reduction of Rubredoxin. Earlier studies (Lovenberg and Sobel, 1965) indicated that rubredoxin is readily reduced by sodium hydrosulfite and that this reduction is accompanied by a loss of the visible absorption. It was of interest, however, to examine the redox properties of rubredoxin crystals. The addition of sodium hydrosulfite to a saturated solution of $(NH_4)_2SO_4$ containing rubredoxin crystals causes them to undergo a progressive bleaching indicating the protein was undergoing reduction in the crystalline form. The photomicrograph in Figure 3 shows that a rubredoxin crystal changes from a deep red to a colorless crystal and can be reoxidized by air after autooxidation of the excess sodium hydrosulfite. Since this complete redox cycle takes place without apparent damage to the crystal it is possible that no major conformational changes are taking place in the protein.

Examination of the optical rotatory dispersion spectrum in the oxidized and reduced state (Figure 4) shows similar changes in the visible portion of the spectrum to those noted previously (Lovenberg, 1966; Gillard *et al.*, 1965). The trough at 233 mμ, which is often attributed to the secondary structure of the proteins, appears to undergo a significant increase in levorotation, suggesting the possibility of a small conformational change during reduction. Because of the optical rotatory properties of the chromophore, however, it was impossible to obtain meaningful data from Moffitt–Yang plots to calculate the degree of helical content in the oxidized and reduced forms.

Nature of the Cysteine Residues in Rubredoxin. Amino acid content of *C. pasteurianum* rubredoxin has been reported previously from this laboratory (Lovenberg, 1966). Preliminary to studying the role of amino acid residues in the iron binding mechanism, the amino acid and iron composition was reexamined together with the extinction coefficients of the protein. The effects of various lengths of hydrolysis on the apparent amino acid content is shown in Table I. Only slight differences were noted in amino acid content at various times of hydrolysis and the proposed amino acid compostion (Table I) is in essential agreement with earlier work (Lovenberg, 1966). From the current results, however, it appears likely that *C. pasteurianum* rubredoxin contains 11 rather than 12 aspartate residues. The cysteine content of rubredoxin has been confirmed as four residues. In two experiments 3.6 and 3.9 moles of cysteic acid were found per mole of performic acid oxidized rubredoxin by amino acid analysis. Approximately 3.9 moles of carboxymethylcysteine were found per mole of alkylated aporubredoxin (Table II). Alkaline hydrolysis of duplicate samples of 48 mμmoles of rubredoxin yielded 91 and 93 mμmoles of tryptophan by fluorescence analysis. A similar hydrolysate yielded 85 mμmoles of tryptophan as determined by use of an amino acid analyzer. This would confirm the presence of two residues of tryptophan per mole of protein.

TABLE I: Estimated Amino Acid Residues per Mole of Rubredoxin after Various Periods of Hydrolysis.[a]

	12 hr	24 hr	48 hr	Proposed Residues
Lys	4.1	4.2	4.1	4
Asp	11.0	10.7	11.3	11
Thr	3.0	2.6	2.6	3
Glu	6.1	6.0	6.1	6
Pro	5.4	5.4	5.3	5
Cys (1/2)	2.7	3.0	2.5	4
Gly	6.2	6.2	6.2	6
Val	5.1	4.9	5.1	5
Met	1.0	0.9	0.8	1
Ilu	1.8	2.0	2.0	2
Leu	1.1	1.0	1.1	1
Tyr	2.8	2.7	2.6	3
Phe	2.0	2.1	2.0	2

[a] The amount of protein used for each hydrolysate was estimated from the glutamic acid, valine, isoleucine, and phenylalanine content in the hydrolysates and the assumption that the preliminary assignment (Lovenberg, 1966) of these residues was correct. The residues of each amino acid were then calculated using this combined standard of reference. The values presented in this table are the average of three separate experiments using separate batches of rubredoxin. Hydrolyses were done as described in the text for the various times stated above. Histidine, arginine, serine, and alanine were not detectable (less than 0.1 residue/mole) in any analysis.

TABLE II: Amino Acid Content of Carboxamidomethylrubredoxin.[a]

	mμmoles	Residues/Mole		mμmoles	Residues/Mole
Lys	133	3.9	Val	162	4.8
CM-Cys	133	3.9	Met	26	0.8
Asp	400	11.7	Ilu	61	1.8
Thr	105	3.1	Leu	36	1.1
Glu	200	5.9	Thr	94	2.8
Pro	160	4.7	Phe	68	2.0
Gly	211	6.2			

[a] The residues per mole were determined as described in Table I. The above values represent the average of duplicate amino acid analyses done on a single hydrolysate of CM-rubredoxin. Approximately 0.034 μmole of CM-rubredoxin was hydrolyzed for 20 hr at 110°.

In order to obtain a clear relationship between the extinction coefficient and the iron and amino acid content, two separate batches of rubredoxin were prepared. These three parameters of the two preparations are compared in Table III. The protein concentration of the solutions was established by amino acid analysis of hydrolysates. The extinction coefficients here are based on the protein content and are similar to those reported earlier (Lovenberg and Sobel, 1965) which were based on Folin protein assay. The presence of a single iron atom in clostridial rubredoxin is also confirmed by both iron assay methods.

TABLE III: Extinction Coefficients and Iron Content of Rubredoxin.

	Prepn I	Prepn II
Protein (μmole/ml)[a]	0.334	0.165
Iron (μmole/ml) by *o*-phenanthroline[b]	0.380	0.160
Iron (μmole/ml) by bathophenanthroline	0.390	0.160
Molar extinction coefficients in oxidized form[c]		
280 mμ	21,600	21,100
380 mμ	11,000	10,700
490 mμ	9,000	8,800

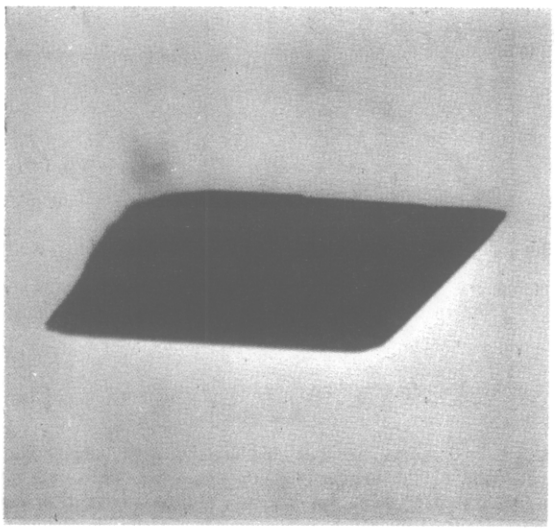
[a] Protein contents of the two rubredoxin preparations were determined by amino acid analysis of aliquots. [b] Iron analyses were done in duplicate using ferrous ammonium sulfate as standard after heating the samples at 80° in 1 ml of 1% HCl for 10 min. [c] The molar extinction coefficients were determined by recording absorption spectra of the appropriate dilutions of the two preparations in 0.05 M Tris-HCl (pH 7.3).

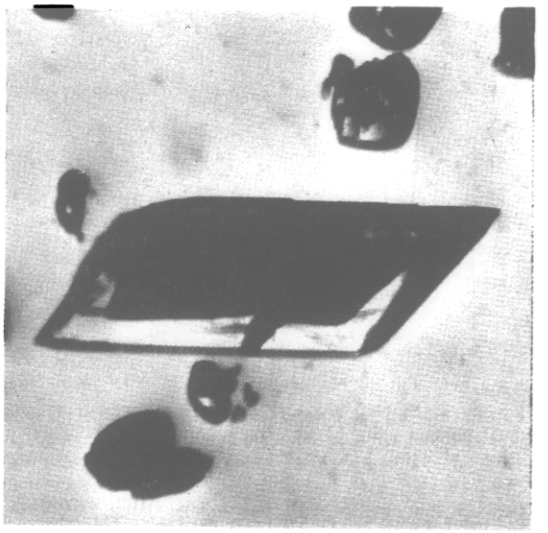
Reaction of Rubredoxin with Mercurials. It was noted in earlier reports that rubredoxins reacted very slowly with *p*-mercuribenzoate (Lovenberg, 1966, 1967) and insignificantly with sodium mersalyl (Lovenberg and Sobel, 1965). A comparison of the rates of reaction of various mercurial compounds with rubredoxin is shown in Figure 5. As shown here sodium mersalyl, the largest of the mercurial compounds studied, reacts with approximately 10% of the cysteine groups during a 10-hr incubation. Mercuric ion reacts most rapidly whereas *p*-mercuribenzoate and *p*-mercuriphenylsulfonate react with intermediate rates. The lack of inorganic sulfide in rubredoxin permits a less complicated interpretation of the role of the cysteine sulfhydryls in the iron binding than is possible in the case of ferredoxin. The reaction of the mercurial with the sulfhydryl groups (ΔOD 250 mμ) is also accompanied by a loss in the visible iron chromophore (ΔOD 490 mμ). It would appear from these and earlier studies (Lovenberg, 1966) that all four half-cysteines are present as SH compounds and all are available for reaction with mercurials.

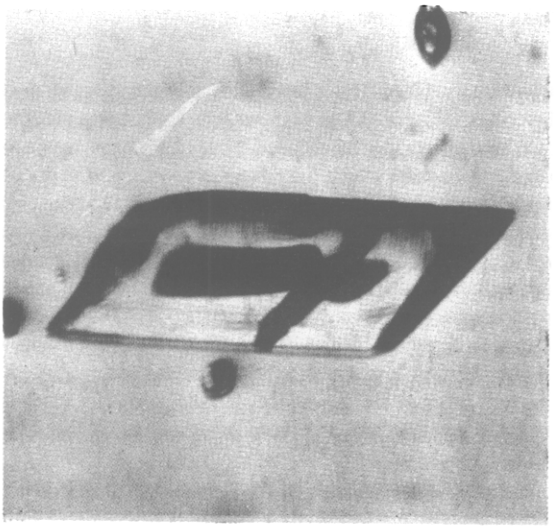
When an excess of mercuric acetate is added to a solution of rubredoxin the loss of absorbancy at 490 mμ occurs in direct proportion to the reaction of four SH groups (Figure 6) with mercurial suggesting that all four SH groups are involved in the metal protein ligands. In these experiments it was also observed that the absorption band at 380 mμ decreases at exactly the same rate as the 490-mμ absorbancy. It is apparent that both these bands are probably associated with the interaction of the cysteine sulfhydryls with the iron.

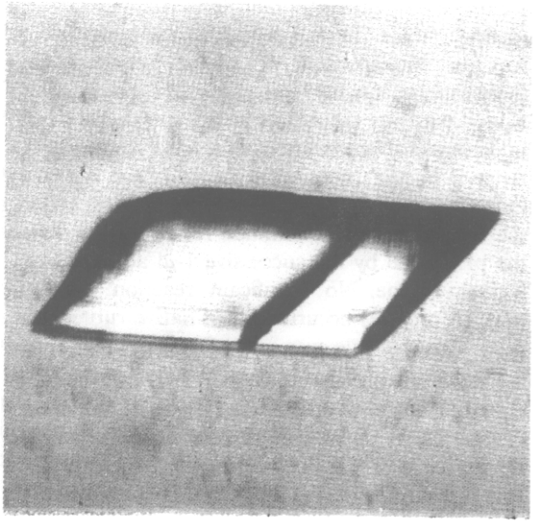
Reaction of Rubredoxin with Iodoacetate. As noted above, reaction of aporubredoxin with iodoacetate

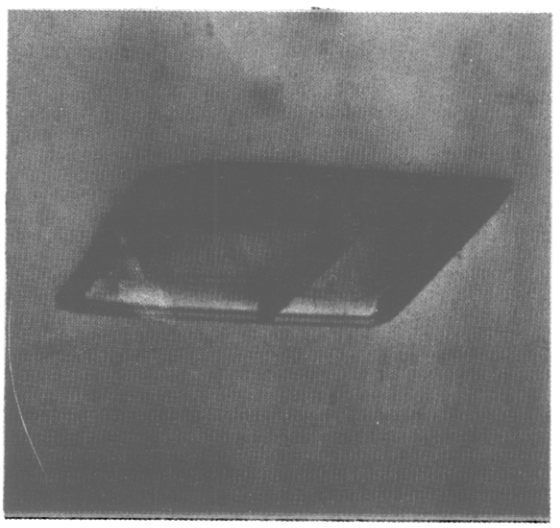
FIGURE 3 (facing page): Reduction and reoxidation of a rubredoxin crystal. A crystal of rubredoxin was suspended in 90% saturated $(NH_4)_2SO_4$ (1 ml), 10–20 mg of sodium hydrosulfite was added, and photomicrographs were then taken of changes occurring in the crystal. (A) (Top left) Oxidized crystal before the addition of sodium hydrosulfite. (B and C) (Top right and middle left) Photographs of the crystal that has been partially reduced. (D) (Middle right) The fully reduced crystal. (E and F) (Bottom left and right) The partially reoxidized crystal. These photomicrographs were taken by Dr. H. C. R. Skinner of Yale University, New Haven, Conn.

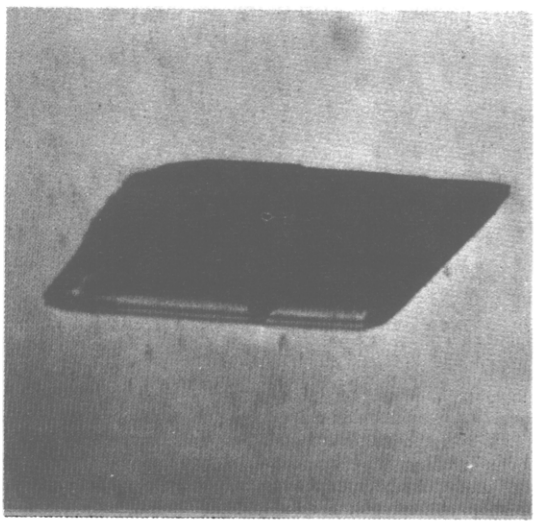












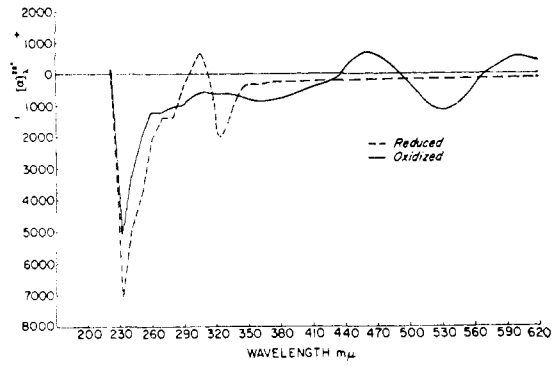


FIGURE 4: Optical rotatory dispersion spectra of oxidized and reduced rubredoxin. The spectra were recorded in an anerobic 5-mm light-path cuvet. The solution contained 1 mM Tris-HCl (pH 7.3) and 0.03 mM rubredoxin and was flushed thoroughly with hydrogen. After recording the oxidized spectra 3 μl of a 1:10 dilution of the hydrogenase preparation was injected. The curves were corrected for the buffer and enzyme blanks.

yielded a protein containing four residues of carboxymethylcysteine. The alkylation of the cysteine residues was also studied using ^{14}C-labeled iodoacetic acid. In these experiments 0.05–0.1 μmole of rubredoxin or aporubredoxin was incubated in 0.5 M Tris-HCl (pH 8.6) under anaerobic conditions in a total volume of 0.6 ml. After 2 hr the tubes were opened, 100 mg of [^{14}C]-iodoacetic acid (2.22×10^4 cpm/μmole) was added, and the incubation was continued for 30 min maintaining the pH at 8.6 by the successive 1-μl additions of 25% trimethylamine. No significant reaction (<0.2 mole/mole of protein) occurred when native rubredoxin was used. This was true even when 8 M urea or 8 M urea and 0.5 M 2-mercaptoethanol were included in the initial incubation. In contrast about 4.2 moles of iodoacetic acid reacted per mole of aporubredoxin when incubated in the presence of 8 M urea and 0.5 M 2-mercaptoethanol. It is therefore apparent that the cysteine sulfhydryls are only available for reaction after complete removal of the iron.

Reconstitution. Addition of iron to a solution of aporubredoxin at neutral pH yielded little or no reconstituted protein. If the pH of the solution was raised to approximately 10 by the addition of trimethylamine and the solution made 0.1 M with respect to 2-mercaptoethanol the formation of colored iron complexes was apparent. A protein which appeared to have spectral properties identical with native rubredoxin was isolated by Sephadex G-25 chromatography when aporubredoxin was treated with ferrous ammonium sulfate and 2-mercaptoethanol at pH 10 (Figures 7 and 8). Under these conditions from 70 to 100% of the aporubredoxin appeared to be reconstituted. In the absence of 2-mercaptoethanol 30% or less of the rubredoxin was reconstituted. The requirement for 2-mercaptoethanol provides further evidence for the probable role of cysteines in the iron binding mechanism.

^{59}Fe-Exchange Studies. In the presence of 2-mercaptoethanol an easily measured exchange of exogeneous iron with the iron of rubredoxin occurs under the conditions used in these experiments. The exchange reaction was

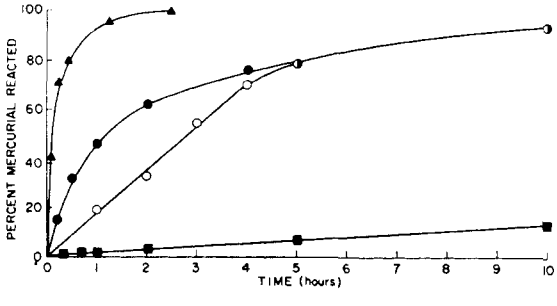


FIGURE 5: Reaction of mercurials with rubredoxin. The reaction was done in a solution containing 10 mM Tris-HCl (pH 6.8), 0.1 mM mercurial, and 0.027 mM rubredoxin. Per cent reaction was measured by changes in absorbancy at 490 mμ. (△) Mercuric acetate, (●) *p*-mercurisulfonate, (○) *p*-mercuribenzoate, and (■) sodium mersalyl.

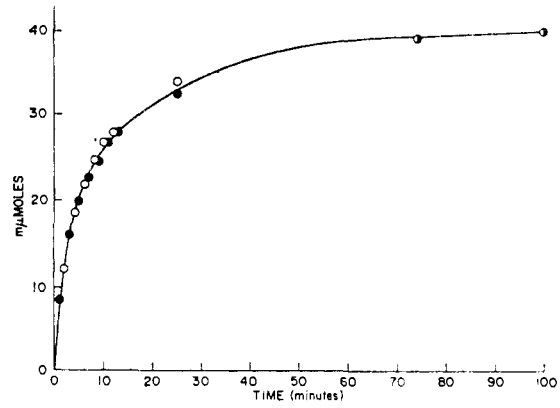


FIGURE 6: Reaction of mercuric acetate with rubredoxin. Formation of the mercuric-sulfur bond was measured by increases in absorbancy at 250 mμ, and destruction of the iron chromophore by decreases in absorbancy at 490 mμ. The extinction coefficient at 250 mμ of the mercuric-sulfur bond (ϵ 3.3×10^3 M^{-1} cm^{-1}) in rubredoxin was calculated from ΔA_{250} when rubredoxin was treated with an excess of mercuric acetate. (●) Δ(Hg-S)/4 and (○) Δ rubredoxin (A_{490} mμ).

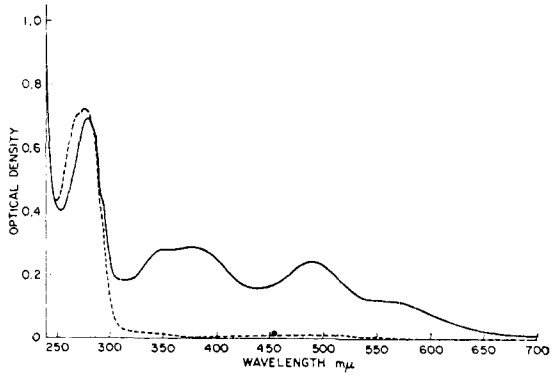


FIGURE 7: The absorption spectra of apo and reconstituted rubredoxin. The spectra were measured in 0.05 M Tris-HCl (pH 7.3). The protein concentrations were approximately 0.033 mM rubredoxin and 0.038 mM aporubredoxin. (- - - -) Aporubredoxin and (——) reconstituted rubreodoxin.

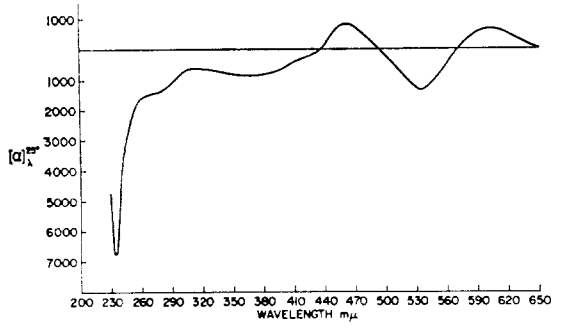


FIGURE 8: The optical rotatory dispersion spectrum of reconstituted rubredoxin. A solution of rubredoxin (approximately 0.02 mM) in 1 mM Tris-HCl (pH 7.3) was used for the spectrum in a cuvet with a 5-mm light path.

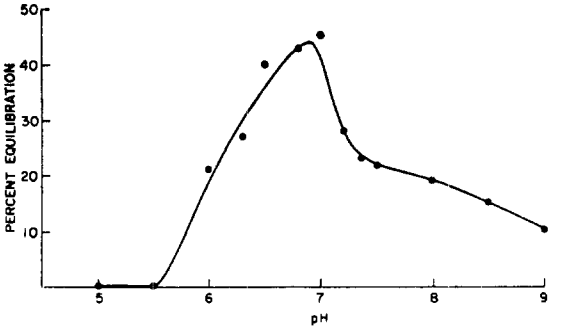


FIGURE 10: Effect of pH on the ^{59}Fe exchange with rubredoxin. Details of the exchange reaction are described in the text.

proportional to time for about 30 min and was essentially complete within 60 min (Figure 9). The exchange reaction appeared to have a sharp pH optimum (Figure 10). The pH curve is very reproducible under the conditions used. The lack of any known ionizable groups in the protein with pK values in the range of pH 7 prohibit any ready interpretation of the unusual pH effects.

The iron exchange was completely dependent upon the presence of 2-mercaptoethanol in the system. This requirement was not only involved in maintaining the iron in the reduced state but also apparently interacted with the protein. Other reductants such as ascorbic acid and/or hydroxylamine could not replace 2-mercaptoethanol (Table IV) and in fact were even inhibitory in high concentrations in the presence of 2-mercaptoethanol. Exchange studies using ferric-59 citrate indicated that iron is adsorbed readily onto rubredoxin; however, no exchange with the iron of the active center appears to take place since most of this adsorbed iron can be easily removed by the addition of 1,10-phenanthroline before the chromatography step.

Discussion

One of the major problems in the isolation of rubredoxin from *C. pasteurianum* has been obtaining the complete separation of the protein from ferredoxin. Both these proteins are small, acidic proteins which normally adhere tightly to DEAE-cellulose and cannot be clearly separated by normal positive gradient elution. While the rationale for the adsorption of these proteins from concentrated $(NH_4)_2SO_4$ solution is not clear the technique of a negative gradient elution reported here was extremely useful in quickly obtaining pure preparations of rubredoxin.

The finding that sodium hydrosulfite completely reduces intact crystals of rubredoxin indicates that this reducing substance readily penetrates the lattice structure of the crystal. Other compounds have also recently been observed to penetrate rubredoxin crystals, and indeed L. C. Sieker and L. H. Jensen (unpublished observations, 1968) find the chloroplatinate can be used to make isomorphous derivatives of rubredoxin crystals. The ability of the crystal to undergo reduction and reoxidation with little or no apparent damage is interesting in view of the possible changes in conformation of the protein during the redox cycle. As has been pointed out recently (Bachmayer *et al.*, 1968a), the probable extension of the optical activity of the iron chromophore into the ultraviolet region rules out any direct correlation of changes in the 233-mμ trough being associated with changes in secondary structure.

The amino acid content of several bacterial rubredoxins has recently been compared (Bachmayer *et al.*,

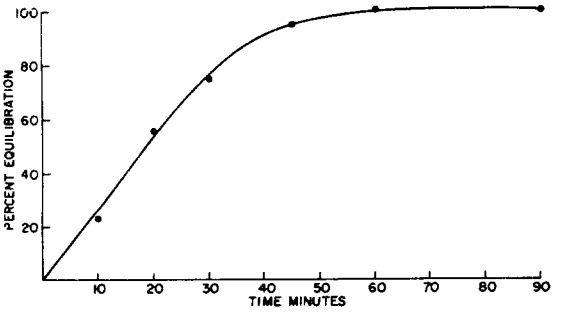


FIGURE 9: Rate of ^{59}Fe exchange with rubredoxin. Experiments were conducted as described in the text.

TABLE IV: Reductants in the Rubredoxin-^{59}Fe Exchange Reaction.[a]

Addition or Omission (μmole)	% Equilibration/20 min
Complete system	45.0
− 2-Mercaptoethanol	<0.4
+ *o*-Phenanthroline	<0.4
− 2-Mercaptoethanol + ascorbic acid (0.01)[b]	<0.4
+ Ascorbic acid (0.01)[b]	40.3
+ Ascorbic acid (0.08)[b]	7.4

[a] These studies were done at pH 6.5 using 0.012 μmole of rubredoxin by the technique described in the text. [b] This ascorbic acid was mixed with tracer $^{59}FeCl_3$ before the addition of carrier ferrous ammonium sulfate.

1968a). It would appear significant that all rubredoxins studied to date contain four half-cysteines and that in the two amino acid sequences determined thus far the cysteines are in identical locations. It is likely that the positioning of the cysteine residues in the polypeptide chain is crucial for the iron binding since it is now clear that the cysteine sulfhydryls are involved in the chromophore of the protein (Lovenberg, 1966, 1967).

The evidence presented in this report further substantiates the participation of the sulfhydryl groups of the protein in the iron binding. The presence of iron in the molecule appears to prevent the reactivity of the sulfhydryl groups of the protein with iodoacetate even in the presence of a reducing compound. Thus it is likely that sulfhydryl groups are not exposed by the reductive process. Experiments such as these however cannot distinguish between "buried" or "blocked" sulfhydryl groups, since undoubtedly the conformation of the protein changes upon release of the iron. Studies with mercurials also suggest that the sulfhydryl groups are not readily accessible in the native protein, since the larger mercurial compounds react extremely slowly with the protein. In spite of this it seems likely from the present data and previous work (Lovenberg, 1966; Bachmayer *et al.*, 1967a) that all four cysteine sulfhydryls are serving as ligands for the iron atom. It is also probable that interaction of the positively charged iron atom with the sulfhydryl would lower the pK values of these groups. This would perhaps account for the anomalous pH curve seen for the iron-exchange reaction.

The requirement for 2-mercaptoethanol in the exchange reaction and the reconstitution also points up the sulfhydryl role in iron binding. It is likely that the requirement for a sulfhydryl reagent and a high pH for reconstitution is due simply to the need to reduce the disulfide bonds which may have been formed during the vigorous treatment used to prepare the aporubredoxin. The ability of the iron of rubredoxin to exchange readily with exogenous iron is further evidence that the iron binding site is distinctly different than that in clostridial ferredoxin (Lovenberg *et al.*, 1963).

A recent report (Bachmayer *et al.*, 1968b) presents evidence that tyrosine and tryptophan may also participate in the iron binding. Further physicochemical studies however will be required to characterize completely the active center of rubredoxin.

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