RUBREDOXIN: A NEW ELECTRON TRANSFER PROTEIN FROM CLOSTRIDIUM PASTEURIANUM

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The nature and role of nonheme iron proteins which participate in a variety of biological oxidations has recently come under intensive scrutiny. Such study received considerable impetus from the discovery and characterization of ferredoxins.[1–3]

During the isolation of ferredoxin a red protein has been observed in extracts of *Clostridium pasteurianum.*[4, 5] We have now isolated this protein in crystalline form and tentatively named it rubredoxin.[6] It is a small nonheme iron protein which can substitute for ferredoxin as an electron carrier in several reactions mediated by extracts of *C. pasteurianum.* Rubredoxin differs from the ferredoxins spectrally and chemically. The properties of purified rubredoxin are the subject of this communication.

Materials and Methods.—Organisms: C. pasteurianum was grown on sucrose utilizing a modification[3] of Carnahan and Castle's medium[7] with ammonium sulfate as the sole nitrogen source. *Clostridium tetanomorphum* was grown on sodium glutamate.[8]

Materials: Ferredoxin-TPN reductase was prepared from fresh spinach leaves as described by Shin *et al.*, and processed through the second DEAE cellulose column step.[9] Clostridial extracts free of ferredoxin and rubredoxin were prepared from sonicated cell suspensions centrifuged at 35,000 $\times$ g for 20 min and applied to a DEAE cellulose column.[1] Other materials were obtained commercially.

Isolation of rubredoxin: Crystalline rubredoxin has an absorbancy ratio (280/490 mμ) of 2.4. Throughout the purification procedure, the degree of purity was ascertained by following optical density at these wavelengths. Initial steps used in the purification were those of Mortenson[5] and may be summarized as follows. Frozen cells are thawed and suspended in 2 vol of distilled water, cooled to 0°, and diluted 1:1 with acetone at 0°. After 15 min, the suspension is centrifuged at 15,000 $\times$ g for 15 min. The supernatant fraction is then applied to a DEAE cellulose column (10 ml of resin bed per 100 gm of frozen cells) which has been previously equilibrated with 1 M potassium phosphate buffer pH 6.5 and subsequently washed with 10 vol of water. Ferredoxin and rubredoxin adhere tightly to the top of the column, which is then washed with 5 vol of water followed by 0.15 M Tris-HCl until the optical density of the effluent at 260 mμ is less than 0.1. Elution of the adsorbed ferredoxin and rubredoxin is accomplished with 0.8 M Cl^- buffer (0.15 M Tris-HCl pH 7.3 containing 0.65 M NaCl). The eluate is desalted on Sephadex G-25.

Ammonium sulfate fractionation: The eluate from the Sephadex column is adjusted to 0.05 M concentration with respect to Tris-HCl pH 7.3 and then made 60% saturated with ammonium sulfate. Rubredoxin and ferredoxin remain in the supernatant fraction which is brought to 90% saturation. At this juncture the precipitate contains most of the ferredoxin which is approximately 95% pure, while the supernatant fraction contains some ferredoxin and most of the rubredoxin.

Second DEAE cellulose column step: The 90% ammonium sulfate saturated supernatant fraction is concentrated by application to a DEAE cellulose column (2 ml of resin bed per 100 gm of original cells) to which the ferredoxin and rubredoxin adhere tightly. Both the electron carrier proteins are eluted directly with the 0.8 M Cl^- buffer.

Second ammonium sulfate fractionation: Again the eluate is desalted, made 0.05 M with respect to Tris-HCl pH 7.3, and saturated to 90% with ammonium sulfate. The precipitate from this fraction contains most of the remaining ferredoxin and is discarded. The supernatant fraction, containing rubredoxin, is readsorbed on a small DEAE cellulose column (2 ml of resin bed usually being sufficient). Elution of rubredoxin is accomplished with the 0.8 M Cl^- buffer, desalting with G-25 Sephadex, and concentration by lyophilization.

Crystallization: The lyophilized rubredoxin is dissolved in water at a concentration of 2–5 mg/ml and precipitated by bringing the ammonium sulfate concentration to 70% saturation. The precipitate is then redissolved in water (5 mg/ml) and the resultant solution brought to 50% saturation with ammonium sulfate. Large red crystals form after several hours at 4°. Rubredoxin can be recrystallized repeatedly by this procedure. The purified protein appears homogeneous by ultracentrifugation and disc gel electrophoresis.

Additional methods: Hydroxylamine reductase activity of clostridial extracts was measured using TPNH as the primary reductant, ferredoxin-TPN reductase from spinach as a mediator of reduction of the electron carrier (ferredoxin or rubredoxin), and hydroxylamine as the oxidant. TPNH oxidation in anaerobic cuvettes under these conditions was measured spectrophotometrically. This oxidation was found to be entirely dependent on the presence of hydroxylamine as a substrate.

Iron and inorganic sulfide analyses were performed as previously described[3] by modifications of the methods of Harvey *et al.*[10] and Fogo and Popowsky,[11] respectively.

Protein was determined by a modification of the phenol reagent assay.[12]

Amino acid analysis: Rubredoxin was oxidized with performic acid[13] and hydrolyzed with 5.7 N glass-distilled HCl at 110° for 20 hr. Subsequently, amino acid content (except for tryptophan) was determined with a Phoenix amino acid analyzer model K 5000. Tryptophan content was determined by fluorescence analysis after alkaline hydrolysis of a sample of the nonoxidized protein.[14]

Results.—Crystallization of rubredoxin: Approximately 1 mg of crystalline rubredoxin was obtained per 100 gm of *C. pasteurianum*. Hexagonal, ruby crystals were obtained and found to be birefringent and approximately 0.1 mm in length on each side (Fig. 1).

Molecular weight: A sample of rubredoxin (0.2 mg/ml in 0.1 M NaCl), centrifuged at 52,640 rpm for 16 hr at 4° in a Spinco model E ultracentrifuge, was used to determine molecular weight by sedimentation equilibrium. The partial specific volume of rubredoxin was not measured, but the approximate molecular weight de-

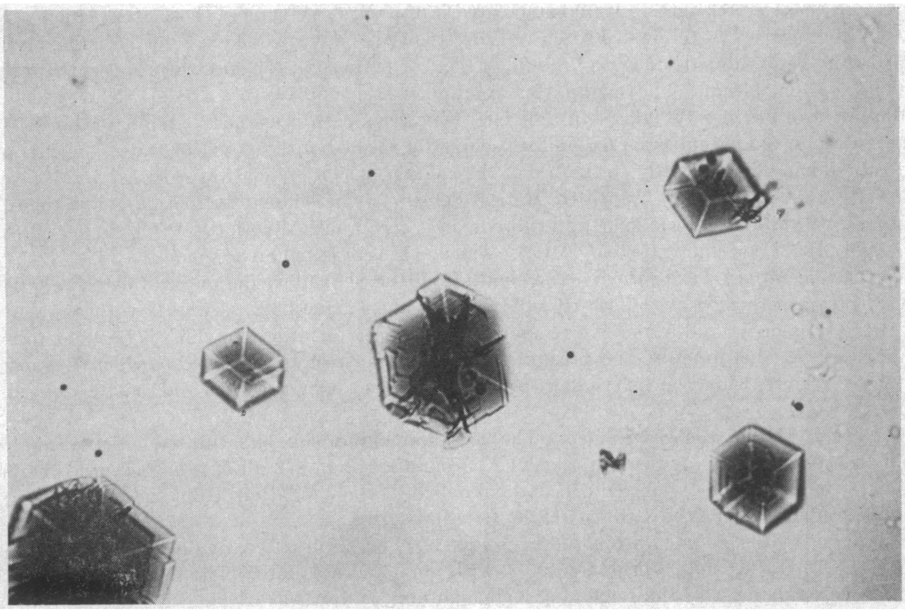

FIG. 1.—Photomicrograph of crystallized rubredoxin from *C. pasteurianum*.

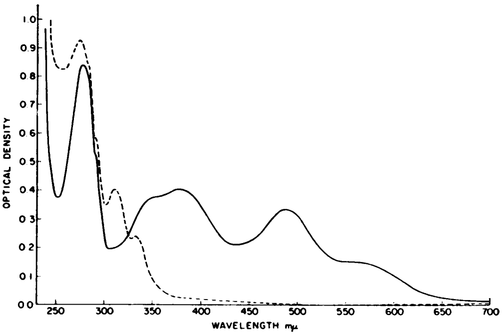

FIG. 2.—Absorption spectra of oxidized (——) and reduced (- - - -) *C. pasteurianum* rubredoxin. The spectra of rubredoxin, 0.23 mg/ml, were measured in 0.1 *M* Tris-HCl, pH 7.0, in a hydrogen atmosphere with a Cary 15 spectrophotometer. The reduced spectrum was obtained after the addition of 20 μl of DEAE-cellulose treated *C. pasteurianum* extract, 5 mg protein/ml, containing active hydrogenase.

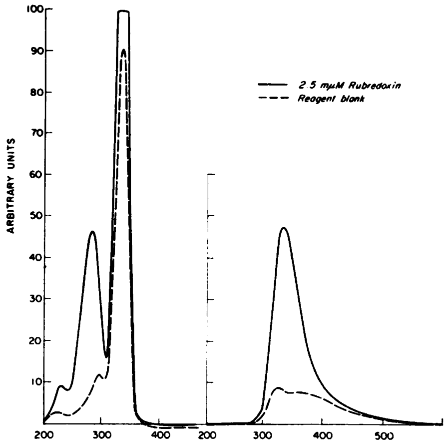

FIG. 3.—Excitation and fluorescence spectra of *C. pasteurianum* rubredoxin. The spectra of rubredoxin, 0.0025 μmoles/ml, were measured in 0.05 *M* phosphate, pH 7.0, in an Aminco-Bowman spectrophotofluorometer.

termined for $\bar{v}$ = 0.70 was 7,200, and for $\bar{v}$ = 0.63 it was 5,900. Thus, rubredoxin has a molecular weight similar to that of clostridial ferredoxin.[3]

Spectral studies: The absorption spectra of oxidized and reduced crystalline rubredoxin are shown in Figure 2. Maxima at 490, 380, and 280 mμ with molar extinction coefficients of 8.85×10^3, 10.8×10^3, and 21.3×10^3, respectively, occur in the oxidized form (the value for molecular weight used in these calculations was 6,000). The reduced form has maxima at 333, 311, and 275 mμ with molar extinction coefficients of 6.3×10^3, 10.8×10^3, and 24.8×10^3 at these wavelengths. Rubredoxin, though auto-oxidizable in air, can be readily reduced by sodium hydrosulfite, hydrogen and *C. pasteurianum* hydrogenase, pyruvate and *C. pasteurianum* pyruvate dehydrogenase, or TPNH and ferredoxin-TPN reductase from spinach.

Rubredoxin has fluorescence properties of a typical tryptophan-containing protein. The excitation and fluorescence spectra are presented in Figure 3. It is of interest that the fluorescence yield is only 10 per cent of that obtained with solutions containing equivalent amounts of free tryptophan. This is due, perhaps, to the strong absorbancy of rubredoxin in the region of 340 mμ.

Determination of the number of equivalents (N) of electrons transferred per mole of rubredoxin: The following system was utilized to determine *N* stoichiometrically.[15] TPNH, rubredoxin, 0.1 *M* Tris pH 7.0 in cuvettes with double-chambered stoppers were made anaerobic by bubbling with prepurified nitrogen for 1 hr. Thirty μl of spinach extract (5 mg/ml) with active ferredoxin-TPN reductase were equilibrated with nitrogen in the inner chamber of the stopper for 30 min. The reaction was initiated by perforating the diaphragm separating the inner chamber from the body of the cuvette and thereby introducing the enzyme into the reaction mixture anaerobically. Optical densities at 325 and 490 mμ were followed. Examination of the spectra of oxidized and reduced rubredoxin (Fig. 2) revealed 325 to be an isosbestic point. Spectral studies of TPNH showed that the ratio of optical density at 340/325 mμ was 1.16. Using these data it is possible to calculate the ratio of moles of rubredoxin reduced per mole of TPNH oxidized.[16] Since two equivalents of electrons are transferred per mole of TPNH oxidized, N for rubredoxin was calculated from the equation:

$$N = \frac{2 \times \text{(moles of TPNH oxidized)}}{\text{moles of rubredoxin reduced}}.$$

The results of typical experiments are shown in Table 1. Since the presence of oxygen in the system constitutes the largest source of error (tending to lead to an erroneously high value of N) and since 1 is both the nearest integral value and a slightly lower value than the actual result obtained, we can be confident that N is equal to 1.

Determination of the redox potential of rubredoxin: Utilizing equilibrium reactions and spectrophotometric measurements,[16] an effort was made to calculate the redox potential of rubredoxin at pH 7.0 (E_0').

Preliminary experiments demonstrated that ferredoxin-TPN reductase was capable of mediating the rapid reduction of indigotrisulfonate by TPNH and of rubredoxin by TPNH under anaerobic conditions. (Indigotrisulfonate was found to obey Beer's law at the pH and in the concentration range used in these studies.) Per cent reduction of rubredoxin and of the dye was determined spectrophotometrically after equilibrium was attained in an anaerobic system containing 0.1 μmoles rubredoxin, 0.09 μmoles dye,[17] 0.157 μmoles TPNH, and 300 μmoles Tris-HCl buffer pH 7.0 in 3 ml. Thirty μl of ferredoxin-TPN reductase (5 mg/ml) were introduced anaerobically to initiate the reaction. Measurements of optical density at 490 and 605 mμ after extinction coefficients for each species had been determined were used to calculate per cent reduction of rubredoxin and of the dye.

The following form of the Nernst equation was used to calculate E_0' for rubredoxin (rb):

$$E_0'rb + \frac{RT}{NF} \ln \frac{\text{oxidized } rb}{\text{reduced } rb} = E_0' \text{ dye} + \frac{RT}{NF} \ln \frac{\text{oxidized dye}}{\text{reduced dye}}.$$

$N = 1$ for rubredoxin (*vide supra*), $N = 2$ for the dye;[18] E_0' dye $= -0.081$ v at pH 7.0.[18] The per cent reduction of rubredoxin and of indigotrisulfonate at equilibrium was 61 per cent and 28 per cent, respectively. Such values lead to a calculated redox potential (E_0' at pH 7.0) of rubredoxin of -0.057 v at 25°.

TABLE 1

DETERMINATION OF THE NUMBER OF EQUIVALENTS (N) OF ELECTRONS TRANSFERRED PER MOLE OF RUBREDOXIN

	Initial OD		OD at Equilibrium		Observed $\Delta \times 3$*		Calculated total	μMoles TPNH	μMoles rubredoxin	
	325 mμ	490 mμ	325 mμ	490 mμ	325 mμ	490 mμ	Δ340 mμ†	oxidized	reduced‡	N
Expt. 1	0.233	0.186	0.178	0.024	0.165	0.486	0.192	0.0297	0.0549	1.1
Expt. 2	0.702	0.591	0.488	0.018	0.642	1.719	0.743	0.120	0.195	1.23

* Final volume = 3 ml.
† See text; 1.16 × Δ325 mμ, total = calculated total Δ at 340 mμ.
‡ μMoles of rubredoxin reduced were calculated as follows, since reduced rubredoxin has no absorbancy at 490 mμ.

$$\text{Per cent reduction} = \frac{OD_{490}\text{, initial} - OD_{490}\text{, at equilibrium}}{OD_{490}\text{, initial}}$$

μmoles reduced = % reduction × (μmoles initially present).

The molar extinction coefficient for rubredoxin at 490 was determined and found to be 8.85×10^3 at pH 7.0. The molar extinction coefficient used for TPNH was 6.2×10^3.[22]

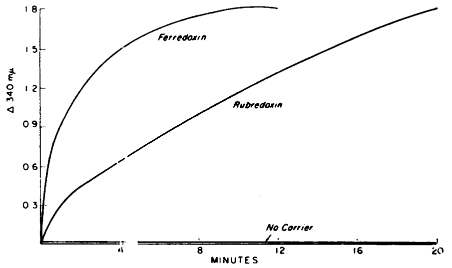

FIG. 4.—Comparison of hydroxylamine reduction mediated by ferredoxin and rubredoxin from *C. pasteurianum*. The complete system contained the following: Tris-HCl, pH 7.0, 300 μmoles; neutralized NH_2OH, 10 μmoles; TPNH, 1 μmole; ferredoxin-TPN reductase, 0.25 mg protein; DEAE cellulose treated *C. pasteurianum* extract, 20 μl containing 0.16 mg protein; and 0.025 μmoles of either rubredoxin or ferredoxin. The final volume was 3 ml.

Some oxidation-reduction reactions mediated by rubredoxin: Valentine *et al.*[19] reported that ferredoxin is required for TPN reduction by hydrogen in ferredoxin-free extracts of *C. pasteurianum*. In equimolar amounts rubredoxin can substitute for ferredoxin in this reaction, but with a resultant reaction rate of only 5 per cent. Although the system exhibits a lack of specificity with respect to the protein electron carrier, it is not surprising that the rate is markedly lower with rubredoxin in view of its higher redox potential. Another system, one in which rubredoxin functions more efficiently, is the hydroxylamine reductase system[20] (Fig. 4). In these systems TPNH and ferredoxin-TPN reductase are used as the source of reducing power.

Because of its low redox potential,[21] ferredoxin is only 20 per cent reduced at the beginning of the hydroxylamine reduction experiments. (This was determined in several experiments with the complete system and in other experiments with TPNH, ferredoxin-TPN reductase, and ferredoxin alone.) As TPNH is oxidized, the per cent of reduced ferredoxin in the system continually decreases, thereby accounting for the constantly decreasing rate of reaction. Conversely, when rubredoxin functions as the carrier, it is virtually completely reduced during the entire course of the reaction because of its higher redox potential, and a constant rate of reaction is observed.

Despite the fact that rubredoxin participates in several biological oxidation-reduction reactions in which it can replace ferredoxin *in vitro*, none of the reactions studied to date appears to represent a specific role for the protein. Additional study is required to ascertain its physiological function.

Chemical characteristics of rubredoxin: Several experiments demonstrated that rubredoxin contains 0.17 μmoles of nonheme iron per mg of protein. Thus, it apparently contains 1 mole of iron per mole.

Rubredoxin was analyzed also for the presence of inorganic sulfide.[3] None was detected. Because rubredoxin is not readily denatured under the conditions usually employed in this assay, it is possible that if inorganic sulfide were present, it might escape detection by virtue of remaining bound to the protein. Therefore, repeat analyses were performed at 80°, a temperature demonstrated to denature rubredoxin. No inorganic sulfide was detected even under the latter conditions. In independent experiments, the assay system itself was validated at 80°.

Since it has been previously suggested[5] that rubredoxin might possibly be a cobamide protein, approximately 400 gm of *C. pasteurianum* cells were isolated from 80 liters of medium containing 1 μc per liter of cobalt 60. The rubredoxin extracted from these cells demonstrated no significant incorporation of radioactivity. It therefore seems clear that rubredoxin is not a cobamide protein.

TABLE 2

AMINO ACID CONTENT OF PERFORMIC ACID OXIDIZED RUBREDOXIN COMPARED TO THAT OF FERREDOXIN

Amino acid	μMoles Amino Acid Sample 1	Sample 2	Proposed Residues per Mole Rubredoxin	Ferredoxin[3]
Lysine	0.788	1.11	4	1
Histidine	n.d.*	n.d.	0	0
Arginine	n.d.	n.d.	0	0
Cysteic acid	0.600	1.05	4	8†
Aspartic acid	2.042	3.08	12	8
Methionine sulfone	0.157	0.215	1	0
Threonine	0.518	0.701	3	1
Serine	0.042	0.038	0	5
Glutamic acid	1.08	1.64	6	4
Proline	0.984	1.35	5	3
Glycine	1.078	1.61	6	4
Alanine	0.037	0.043	0	8
Valine	0.815	1.34	5	6
Isoleucine	0.350	0.575	2	5
Leucine	0.155	0.280	1	0
Tyrosine	0.198	0.177	1	1
Phenylalanine	0.375	0.545	2	1
Tryptophan	...	...	2	0

* None detectable.
† Unpublished observations of Raftery, Lovenberg, and Cole.
Amino acid composition was determined as described in the *Methods* section. Samples 1 and 2 were obtained from separate preparations and were found to contain 0.24 and 0.22 μmoles of rubredoxin, respectively, before the performic acid oxidation step was performed.
In two separate preparations of rubredoxin (see text), the following values for μmoles of tryptophan per μmole of rubredoxin were obtained: 2.05 and 2.04.

The amino acid content of rubredoxin was determined (Table 2). It is interesting to note that several amino acids are absent, and that, as is the case with ferredoxin, the molecule contains a preponderance of acidic amino acids. Although rubredoxin and ferredoxin are of similar size, striking differences in amino acid content are observed. Two of the most prevalent amino acids in *C. pasteurianum* ferredoxin, alanine and serine, are entirely lacking from rubredoxin; while methionine and tryptophan, present in rubredoxin, are lacking from ferredoxin. All clostridial ferredoxins examined to date[3] contain either zero or one basic residue. Rubredoxin, however, contains four residues of lysine. Fortuitously, because of the distinct amino acid content of each of these two proteins, amino acid analysis serves to ascertain the purity of each with respect to the other. This is especially helpful in view of the similar isolation procedures employed in purifying both proteins.

Rubredoxin undergoes very slow bleaching of the visible spectrum with sodium mersalyl. In contrast, ferredoxin reacts within several seconds under identical conditions. The bleaching that does occur is not reversible with 2 mercaptoethanol in contradistinction to the case with clostridial ferredoxin.[3]

Rubredoxin is remarkably stable in acid conditions at room temperature. It takes approximately 10 hr of exposure to 1 per cent sulfuric acid to produce denaturation characterized by complete loss of absorbancy at 490 mμ. Under the same acid conditions at 80° the loss of the 490 mμ absorbancy occurs within 30 sec.

Discussion.–Interest in nonheme iron proteins has been stimulated by the recent discovery of *Clostridial* ferredoxin and by the demonstration of the similarity[21] between photosynthetic pyridine nucleotide reductase from plants and ferredoxins from bacteria. These acidic proteins contain equimolar amounts of nonheme iron and inorganic sulfide. They have characteristic visible absorption spectra and lack an EPR signal in the range of $g = 1.90$. Considerable interest has been evinced in another group of nonheme iron proteins of which some flavoprotein dehydrogenases are members. These contain equimolar nonheme iron and inorganic sulfide also but have characteristic EPR signals, in the range of $g = 1.90$.[23] Nonheme iron proteins, probably containing inorganic sulfide, have been implicated as functional entities in electron transport in mitochondria from mammalian tissue.[24]

As we have indicated, rubredoxin can be chemically and spectrally distinguished from these other nonheme iron proteins. A striking feature is its lack of inorganic sulfide. Rubredoxin represents, perhaps, a distinct class of electron transfer proteins. It is hoped that the study of rubredoxin will provide insight into functional nonheme iron proteins in a variety of electron transfer systems.

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