Preparation and Properties of Cobalt(II) Rubredoxin†

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ABSTRACT: The native iron atoms in rubredoxin from *Pseudomonas oleovorans* were replaced with cobalt to give rubredoxin containing two atoms of cobalt per protein molecule. This represents the first example of metal substitution by chemical reconstitution in an iron–sulfur protein, and thus a number of properties of the cobalt enzyme were examined. Cobalt rubredoxin was characterized as to metal content, and competitive ligation studies established that the apoenzyme binds cobalt preferentially over iron. The spectral properties of cobalt rubredoxin are consistent with the presence of two Co(II) atoms in a rubredoxin-type binding site, exhibiting d–d transitions and charge-transfer bands of the intensity and position predicted from data with model compounds. The CD, MCD, and resonance Raman spectra were also compared for both native and cobalt rubredoxin. As judged from difference spectra, cobalt rubredoxin interacts with rubredoxin reductase and mediates reduction of cytochrome *c* in the presence of reduced nicotinamide adenine dinucleotide and reductase, although it is less efficient in this regard than native rubredoxin. Cobalt rubredoxin is much more stable than the native enzyme toward denaturants and metal dissociation, and no evidence for nonequivalence of the two cobalt binding sites was obtained. The relative stability of cobalt binding allowed for the first time selective chemical modification of two reactive sulfhydryl groups on rubredoxin, and the modified enzyme exhibited 70% of the activity of unmodified cobalt rubredoxin in electron transfer to cytochrome *c*.

Replacement of the native metal at the active sites of metalloproteins by other transition metals has been a useful technique in identifying metal-binding groups and in probing for alterations in coordination geometry upon binding of inhibitors and substrates. Cobalt is a particularly suitable environmental probe for such purposes due to its paramagnetism and the sensitivity of its visible spectrum, especially in the d–d transition region, to changes in coordination geometry (Vallee and Williams, 1968; Vallee and Wacker, 1970; Lindskog, 1970; Garbett et al., 1972). Metalloproteins in which the native metal has been chemically replaced by cobalt include carboxypeptidase A, carbonic anhydrase, neutral protease, alkaline phosphatase, yeast aldolase, yeast enolase (for review, see Vallee and Wacker, 1970), phosphoglucomutase (Ray et al., 1972), myoglobin, hemoglobin (Hoffman and Petering, 1970), horseradish peroxidase (Wang and Hoffman, 1977), liver alcohol dehydrogenase (Drum and Vallee, 1970; Sytkowski and Vallee, 1976), and stellacyanin (McMillan et al., 1974). Of particular interest are the latter two, which contain one or more sulfur ligands. Studies with the Co(II) analogue of stellacyanin confirmed the notion that the intense blue bands of native stellacyanin are attributable to a Cys-S^- → Cu(II) charge-transfer transition (McMillan et al., 1974). The Co(II) complex $[Co(S_2\text{-}o\text{-}xyl)_2]^{2-}$ has recently been prepared and examined spectrally (Lane et al., 1977), and this synthetic thiolate complex provides a model for Co(II) in a rubredoxin-type metal-binding site.

We report herein on the first preparation of cobalt rubredoxin and on the effect of substitution with the environmentally sensitive cobalt atom on the chemical and physical properties of this enzyme. To our knowledge, this represents the first example of chemical replacement of cobalt for the native metal in an iron–sulfur protein.

Materials and Methods

All inorganic chemicals were of reagent grade or better, and hydrocarbons and organic materials were routinely distilled or recrystallized before use. Preswollen DEAE-cellulose (DE-52) was purchased from Whatman; Sephadex and Sepharose were from Pharmacia; Chelex-100 (100–200 mesh, sodium form) was from Bio-Rad; Aldrithiol-4 (4,4′-dithiodipyridine) and *m*-chloroperoxybenzoic acid were from Aldrich; *p*-hydroxymercuribenzoate was from Sigma; trichloroacetic acid and 1,10-phenanthroline (certified reagents) were from Fisher. Spectrographically pure cobalt chloride and cobalt sulfate were obtained from Johnson Matthey, London, England. All other materials were obtained, synthesized, or purified as previously described (May and Kuo, 1977).

For reconstitution experiments, all buffer solutions used were pretreated either by extracting with dithizone (diphenylthiocarbazone, Fisher) in CCl_4 or by the following procedure (Himmelhock et al., 1966): Chelex-100, which had been treated sequentially with 1 M NaOH, water, and 10 mM Na_4EDTA,[1] was washed with distilled water which had been freed of trace metal contaminants by passage over a mixed-bed ion-exchange column. One volume of the treated Chelex was mixed with 4 volumes of Tris base, stirred for 30 min, and then centrifuged to remove the Chelex. All glassware was cleaned by soaking in 2 N HNO_3 overnight, followed by rinsing in deionized water.

Rubredoxin was isolated from octane-grown cells of *Pseudomonas oleovorans* according to the procedures which we have previously described. The purified protein exhibited normal spectral properties with an A_{280}/A_{497} ratio of 6.6.

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[1] Abbreviations used: (2Fe)- or (2Co)-rubredoxin, rubredoxin containing either two iron or two cobalt atoms per molecule, respectively; CD, circular dichroism; MCD, magnetic circular dichroism; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; NADH, reduced nicotinamide adenine dinucleotide.

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NADH:rubredoxin oxidoreductase [EC 1.6.7.2], hereafter referred to as reductase, was isolated using a new procedure which utilizes DEAE-cellulose chromatography followed by hydrophobic chromatography on aminoalkyl-Sepharose (May and Kuo, in preparation).

All UV-visible spectra were obtained either on an Aminco DW-2 or a Beckman ACTA MVI spectrophotometer, at 25 °C.

Preparation of Aporubredoxin. Rubredoxin was precipitated by trichloroacetic acid in the presence of 0.5 M mercaptoethanol under nitrogen. The precipitate was dialyzed in acetic acid as described by Lode and Coon (1971), and the resulting apoenzyme was lyophilized and stored at −20 °C. The apoenzyme thus obtained exhibited no visible absorbance, indicating loss of the iron chromophore. As a further test for complete iron removal, assay of residual electron-transfer activity was carried out using cytochrome *c* in the presence of rubredoxin reductase and NADH. The iron content of aporubredoxin was further examined by atomic absorption spectrometry to confirm complete iron removal.

Preparation of Reconstituted Rubredoxin. Our method for preparing (2Fe)- and (2Co)-rubredoxin is simpler than that of Lode and Coon (1971) and avoids the possible metal contamination from repeated precipitation by trichloroacetic acid, which usually results in insoluble protein in reconstitution mixtures. The lyophilized apoprotein was dissolved at a final concentration of 5 mg/mL in a 0.5 M Tris base containing 0.06 M mercaptoethanol, and the clear colorless solution was flushed with nitrogen for 5 min. After incubation at room temperature under nitrogen for 3 h, a freshly prepared aqueous solution of spectrographically pure cobalt sulfate or cobalt chloride was added at a molar concentration twice that of aporubredoxin. The green-colored mixture was allowed to stand under nitrogen for 10 min, after which it was readily oxidized by exposure to air to give a light-yellow product [cf. a similar observation with the cobalt(II) complexes of Boc-(Gly-L-Cys-Gly)$_4$-NH$_2$ by Anglin and Davison (1975)]. Sephadex G-25, preequilibrated with 0.05 M Tris-Cl at pH 7.3, was used for desalting at 4 °C. (2Fe)-rubredoxin was prepared by the same procedure but with ferrous ammonium sulfate as the iron source. The reconstitution mixture was pink and deep red, before and after exposure to the air, respectively. A twofold molar excess of either salt with respect to the apoprotein was added to reconstituting mixtures in all cases. A comparative ligation study was also performed with various stoichiometric amounts of both iron and cobalt salts. The iron and cobalt content was measured by atomic absorption (Perkin-Elmer Model 460) at 248.3 and 240.7 nm, respectively. Concentrations of either cobalt or iron rubredoxin were determined spectrophotometrically at 280 nm.

Natural and Magnetic Circular Dichroism Spectra. MCD and CD spectra were obtained at room temperature with a specially devised spectropolarimeter by Dr. R. H. Felton with or without a magnetic field of 16 kG using a solution of 8.3×10^{-5} M cobalt rubredoxin in a cell of 1-cm light path. MCD and CD spectra are additive; hence, all MCD spectra have been corrected for the CD component. Molecular magnetic ellipticity, $[\theta]_M$, is given in units of deg cm^2 dmol^{-1} G^{-1}. A solution of $CoSO_4$ at 0.137 M was used as a standard ($[\theta]_M = 6.2 \times 10^{-3}$ at 510 nm, McCaffery et al. [1967]). Molecular ellipticity for CD spectra, $[\theta]_\lambda$, is given in units of deg cm^2 dmol^{-1}. All spectra were baseline corrected using a buffer blank in the same cell. CD measurements were also performed with a Durrum-Jasco UV-5 recording spectropolarimeter equipped with a CD accessory.

Laser-Raman Spectra. The Raman spectrometer was equipped with a Coherent Radiation CR-5 Ar^+ laser, a Spex 1401 double monochromator, and a cooled RCA C-31034 phototube. Spectra were obtained by Mr. D. Cheung in a rotating cell using transverse-laser (4880 Å) excitation.

TABLE I: Comparison of Iron and Cobalt Binding to Aporubredoxin.

reconstitution conditions; metal present (g-atoms/mol of protein)		reconstituted product metal bound (g-atoms/mol of protein[a])		
Co	Fe	Fe	Co	total metal
0	0	0	0	0
2	0	0	1.95	1.95
2	0.2	0.25	1.91	2.16
2	1.0	0.67	1.48	2.15
2	2	1.14	1.42	2.56
1	2	1.27	0.91	2.18
0.2	2	1.56	0.13	1.69
0	2	1.90	0	1.90

[a] As determined by atomic absorption.

Sulfhydryl Titrations. Concentrations of aldrithiol-4 (4,4′-dithiodipyridine) and PHMB (*p*-hydroxymercuribenzoate) were measured spectrophotometrically using ϵ_M 16 300 M^{-1} cm^{-1} at 247 nm (Grassetti and Murray, 1967) and ϵ_M 16 900 M^{-1} cm^{-1} at 234 nm (Boyer, 1954), respectively. Aldrithiol-4 stock solutions were prepared by dissolving 11 mg of reagent in 0.1 M Tris-Cl at pH 7.3 at 40 °C. For PHMB, stock solutions were prepared by dissolving 8 mg of reagent in 1 mL of 0.04 M NaOH, diluting the solution to 25 mL with water, and then centrifuging to remove particulate material. Prior to use, this stock solution was diluted with 0.01 M sodium-phosphate buffer at pH 7.0. The PHMB solution was stored at room temperature in the dark.

Sulfhydryl titrations were performed spectrophotometrically at 324 nm with Aldrithiol-4 using $\Delta\epsilon_M$ 19 800 M^{-1} cm^{-1} (Grassetti and Murray, 1967) or at 250 nm with PHMB using $\Delta\epsilon_M$ 7600 M^{-1} cm^{-1} (Boyer, 1954).

For total sulfhydryl content after metal removal, (2Fe)-rubredoxin suspended in 1.0 mL of 5 mM Tris-Cl at pH 7.3 containing 1 mM EDTA was denatured in situ by the addition of 10 μL of glacial acetic acid. After 10 min, A_{497} was found to be essentially zero, and the colorless solution was raised to pH 7.5 by the addition of 0.2 mL of 1 M Tris base, and the titrant Aldrithiol-4 was added immediately. The same treatment in the reference cuvette containing buffer solution was also carried out. Background absorption from the protein was corrected.

To determine the effect of modification of the available sulfhydryl groups on electron-transfer activity, two sulfhydryl groups per mole of cobalt rubredoxin were consecutively subjected to PHMB reaction, followed by extensive dialysis against 0.1 M Tris-Cl at pH 7.3 using a UM-10 Amicon ultrafiltration membrane. The modified cobalt rubredoxin was then assayed for cytochrome *c* reduction activity.

Results

Aporubredoxin, obtained by the technique reported here, contains no residual iron, as measured by either atomic absorption spectrometry or electron-transfer activity (Table I; Figure 4). Although native rubredoxin, as isolated, is primarily the (1Fe) species, reconstitution with ferrous ammonium sulfate gives the (2Fe)-rubredoxin with spectral properties which are essentially identical to those reported by Lode and

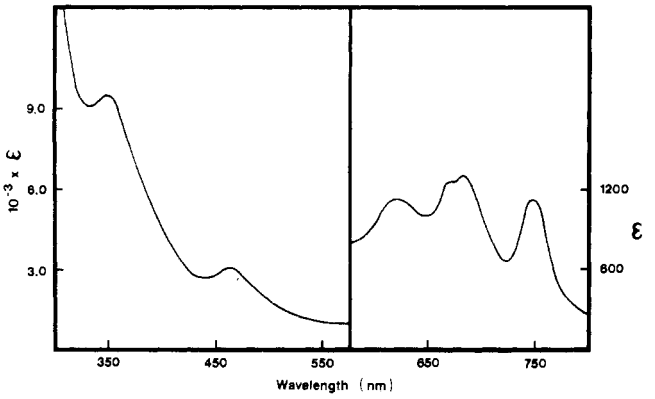

FIGURE 1: Absorption spectrum of cobalt rubredoxin. Average of at least ten preparations in 0.05 M Tris, pH 7.3, 25 °C.

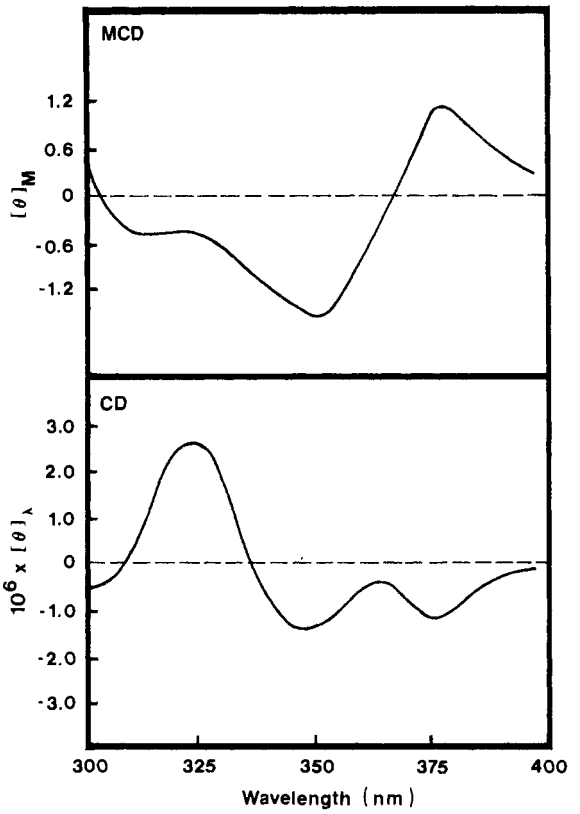

FIGURE 2: MCD and CD spectra of cobalt rubredoxin. The sample concentration was 8.3×10^{-5} M in 0.05 M Tris-Cl at pH 7.3 with a maximum absorption of 0.78 at 350 nm in a path length of 1 cm. The natural CD has been subtracted from the total ellipticity in the magnetic field before normalizing to unit field (magnetic field, 16 kG).

Coon (1971) using a somewhat different reconstitution procedure. The enzyme is fully active in electron transfer from NADH via the flavoprotein reductase to cytochrome *c*. Careful reconstitution of aporubredoxin in the presence of approximately stoichiometric (based on binding sites) amounts of cobalt salts gives cobalt rubredoxin containing 2 g-atoms of cobalt per molecule of protein (Table I). Numerous experiments established that reconstitution in the presence of excess cobalt (e.g., 5:1) gives preparations with altered spectral properties, and atomic absorption data with these species indicated variable, nonspecific binding of cobalt.

As shown in Table I, competitive ligation studies demonstrate preferential binding of cobalt over the native iron atom. The nearly constant total of 2 g-atoms of metal per protein

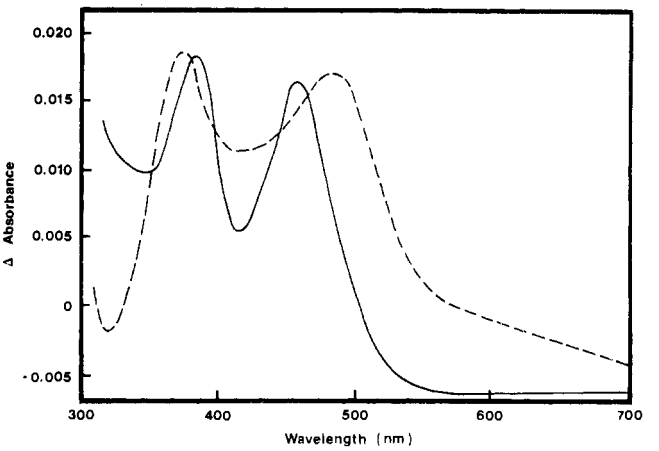

FIGURE 3: Difference spectra of rubredoxin–reductase complexes. The spectrum of a mixture of reductase and (2Fe)- or (2Co)-rubredoxin minus that of the separate components was measured at 25 °C by the use of a split-compartment cell with a 0.45-cm light path in each compartment. The concentrations were rubredoxin, 3.7×10^{-5} M; reductase, 3.3×10^{-5} M, in 0.02 M phosphate buffer: (—) for cobalt rubredoxin; (- - -) for iron rubredoxin.

molecule, despite the wide variations in the composition of the reconstitution mixtures, reflects competition of cobalt and iron for the same binding site.

Spectral Properties of Cobalt Rubredoxin. The spectral properties of cobalt rubredoxin are fully consistent with the presence of two cobalt atoms in a rubredoxin-type binding site (Figure 1). The spectrum exhibits absorption maxima at 350, 470, 620, 685 (splittings), and 748 nm with molar absorptivities (ϵ) of λ_{350} 9405, λ_{470} 3010, λ_{620} 1128, λ_{685} 1232, and λ_{748} 1034, respectively. The intensity and position of the 350-nm band are consistent with charge transfer between Co(II) and thiolate ligands, and are in excellent agreement with those observed in both protein and model systems (ca. 900–1300/Co–S–Cys bond; Curdel and Iwatsubo, 1968; Drum and Vallee, 1970; McMillan et al., 1974; Sytkowski and Vallee, 1976; Lane et al., 1977). The d–d bands in the visible region are as expected for the distorted tetrahedral high-spin Co(II) system (Davison and Reger, 1971; Davison and Switkes, 1971; Anglin and Davison, 1975).

While the CD spectrum which we obtained for the iron species is similar to that reported by Peterson and Coon (1968), exhibiting a number of strong extrema in the visible region, both positive and negative, the cobalt enzyme exhibits weak extrema at 375 and 348 nm and a positive extremum at 322 nm (Figure 2). The corresponding MCD spectrum exhibits a negative Faraday effect at 350 nm, corresponding to the absorption maximum in the visible spectrum, and a positive Faraday effect at 375 nm.

The resonance Raman spectrum of the cobalt enzyme exhibits only two bands at 419 and 343 cm^{-1}, while we observed bands at 365 and 313 cm^{-1} for (2Fe)-rubredoxin. These latter values are in excellent agreement with the spectrum reported by Long and Loehr (1970) for iron-containing clostridial rubredoxin species.

Interaction with Reductase and Electron-Transfer Activity. Difference spectra for the interaction of the oxidized form of cobalt or iron rubredoxin with reductase are shown in Figure 3. It is evident that cobalt rubredoxin interacts with the reductase, with the maxima being shifted somewhat from those which we obtained with the iron enzyme [cf. Ueda and Coon, 1972]. We note the negative differences in the d–d region, possibly indicative of distortions in cobalt coordination ge-

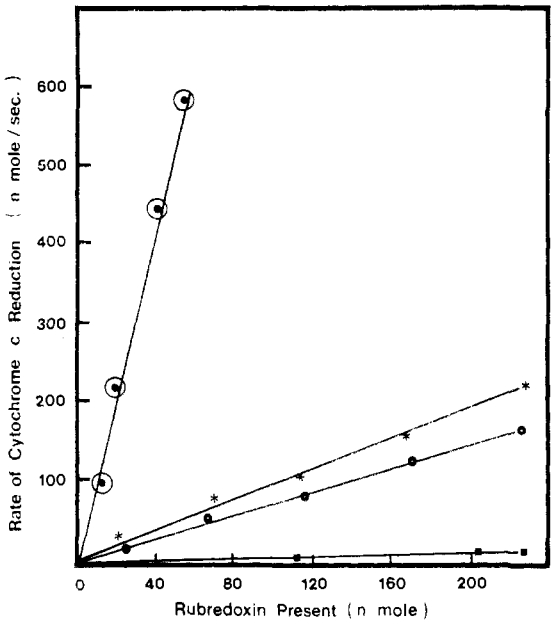

FIGURE 4: Comparative electron transfer activities of (2Fe)-, (2Co)-, PHMB-modified Co-, and aporubredoxin. In each assay, varying amounts of rubredoxin were added to 24 nmol of cytochrome *c* in 1.0 mL of 0.1 M Tris-Cl, pH 7.8, and the mixtures were incubated at 30 °C for 3 min. Rubredoxin reductase (0.8 μmol) and NADH (0.28 μmol) were then added to initiate the reaction. Cytochrome *c* reduction was monitored at 550 nm using $\Delta\epsilon$ 2.1 × 10^4. The rate contributed from the reductase and NADH was subtracted. The modified cobalt rubredoxin had been reacted with 2 mol of PHMB/mol of protein: (●-●) (2Fe)-rubredoxin; (○-○) modified cobalt rubredoxin; (*-*) (2Co)-rubredoxin; (■-■) aporubredoxin.

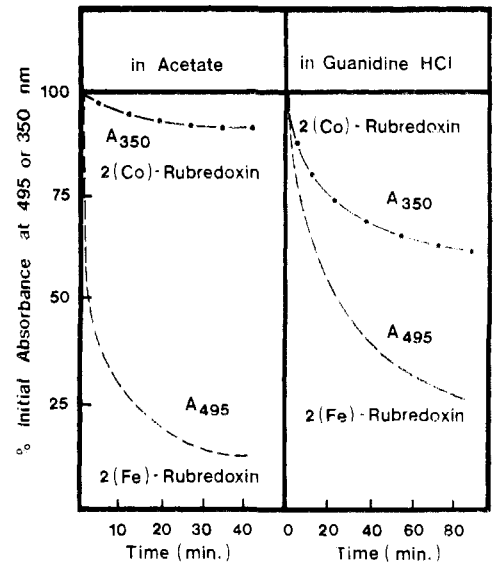

FIGURE 5: Stability comparison of (2Fe)- and (2Co)-rubredoxin. In separate experiments, each protein (0.07 mg) was added to (A, left) acetate buffer to give an acetate concentration of 0.05 M at pH 3.3 and (B, right) guanidine hydrochloride to give a guanidine hydrochloride concentration of 6 M at 30 °C, and absorbances were continuously monitored at the indicated wavelengths.

ometry upon complex formation. The data indicate that cobalt substitution does not result in conformational changes so gross as to prevent complexation with reductase.

The data in Figure 4 establish that cobalt rubredoxin also mediates the reduction of cytochrome *c* in the presence of reductase and NADH, although it is less efficient in this role than is iron rubredoxin (cf. May and Kuo, 1977). Under the conditions of these experiments, the reactions were linear with time, and the rates are linearly dependent on the amount of either rubredoxin species present. For comparison, the activity of aporubredoxin in this concentration range is shown to be negligible, and this establishes a role for cobalt in electron-transfer activity.

Stability toward Chelating and Denaturing Reagents. Native rubredoxin exhibits loss of iron upon prolonged storage, and the "second" iron atom in the (2Fe) species (N-terminal site) is exceedingly labile (Lode and Coon, 1971). In sharp contrast, cobalt rubredoxin (at A_{350} = 0.35) exhibits essentially no spectral change at 4 °C for several weeks. Treatment of (2Fe)-rubredoxin (18 mmol) with a twofold excess of 1,10-phenanthroline at pH 7.3 results in substantial loss of iron, with spectral changes observable within the first few minutes, while under similar conditions only small changes occur in the d-d region of the (2Co) species. Figure 5 illustrates that cobalt rubredoxin is also much more stable than the Fe enzyme toward denaturation in either 6 M guanidine hydrochloride or 0.05 M acetate (pH 3.3). Since loss of absorbance at 495 or 350 nm arises from disruption of the iron or cobalt chromophore, respectively, it is apparent that this process occurs much less readily in cobalt rubredoxin than in the iron enzyme. It is also apparent that, as expected, disruption of iron chromophores is biphasic, the N-terminal site being vastly more labile even in the absence of denaturants. We have obtained no evidence for a similar nonequivalence of cobalt binding in (2Co)-rubredoxin. As expected, only very small changes in the protein absorbance at 280 nm were observed upon treatment of either the cobalt or iron enzymes with denaturants.

Sulfhydryl Titrations. In our hands, titration of aporubredoxin, which had been prepared in situ from (2Fe)-rubredoxin, with Aldrithiol-4 gave reaction of ten cysteine residues, which is consistent with the amino acid composition data (Lode and Coon, 1971). In contrast, Lode and Coon (1971) reported that at most 8.6 cysteine residues could be titrated by Ellman's reagent (Nbs_2). A similar difference between these two reagents toward the thiol groups of urea-denatured thyroglobulin has also been reported (Pitt-Rivers and Schwartz, 1967); Aldrithiol-4 reacted with thiol groups which were inaccessible to Ellman's reagent.

P. oleovorans rubredoxin differs from those of the anaerobes in that it contains two iron-binding sites, and data with the CNBr-cleaved enzyme support the notion that each binding site is composed of cysteine residues from only one end of the molecule (Lode and Coon, 1971). Since the sequence data (Benson et al., 1971) establish that a cluster of five sulfhydryl groups exists near each terminus, a single uncoordinated thiol group is presumably available near each metal-binding site or, alternatively, a diterminal disulfide exists. It has thus been an important goal to probe for two "reactive", non-metal-protected, sulfhydryls using selective chemical modification, in order to test for a possible role in electron transport or catalysis. However, both the data of Lode and Coon (1971) with Nbs_2 and our own work with both Aldrithiol-4 and PHMB (Kuo, J. Y., and May, S. W., unpublished results) establish that the lability of the second iron in (2Fe)-rubredoxin precludes selective modification of two sulfhydryls per protein molecule. In sharp contrast, with (2Co)-rubredoxin, titration with Aldrithiol-4 reveals the presence of two highly reactive sulfhydryl groups. As shown in Figure 6, titration data with PHMB reveals that with PHBM/protein ratios of up to six, only two sulfhydryl groups in the 2Co species react vs. four sulfhydryls in the 2Fe species. Modification of two sulfhydryl groups in the cobalt enzyme is accompanied by small changes (less than 15%) in the visible chromophore. Using the conditions estab-

lished in Figure 6, cobalt rubredoxin with two sulfhydryls modified was prepared on a preparative scale, isolated by ultrafiltration, and assayed for electron-transfer activity toward cytochrome *c* in the presence of NADH and reductase. As shown in Figure 4, the modified enzyme exhibited 70% of the activity of unmodified (2Co)-rubredoxin.

Discussion

Taken together, the data reported in this paper establish that reconstitution of aporubredoxin in the presence of cobalt salts by the method we describe gives cobalt rubredoxin containing two Co(II) atoms per protein molecule, each of which is in a rubredoxin-type binding site. The spectrum of cobalt rubredoxin between 600 and 800 nm exhibits bands corresponding to the spin-allowed ligand field transitions: $^4A_2 \rightarrow {}^4T_1(P)$. The positions and intensities of these bands are indicative of a distorted tetrahedral high-spin Co(II) core (Davison and Reger, 1971; Davison and Switkes, 1971; Anglin and Davison, 1975). The intense band at 350 nm is very likely an $S^- \rightarrow$ Co(II) charge-transfer absorption, in excellent accord with those observed in Co(II) yeast alcohol dehydrogenase, Co(II) liver alcohol dehydrogenase, Co(II) stellacyanin, and a well-defined synthetic Co(II)-thiolate complex $[Co(S_2\text{-}o\text{-}xyl)_2]^{2-}$ (Lane et al., 1977). In each of the above examples, at least one thiolate ligand was coordinated to the cobalt atom. The calculated extinction coefficients were in the range of 900–1300/Co–S–Cys bond. Thus, in the case of cobalt rubredoxin, the extinction of 9405 at 350 nm is consistent with the conclusion that a total of eight Cys-S groups are involved in coordination of the two cobalt atoms.

The assignment of the absorption band at 470 nm is still uncertain. Tentatively, if this is a charge-transfer band and corresponds to the absorption of iron rubredoxin at 495 nm, while that at 350 nm corresponds to the latter at 365 nm (center), then the energy separation between two charge-transfer bands in cobalt(II) rubredoxin of 7295 cm^{-1} is in good agreement with the 7195 cm^{-1} energy separation in (2Fe)-rubredoxin. A rough linear correlation between the charge-transfer band position has been observed for a series of metal ions possessing a given ligand environment (Barnes and Day, 1964). Likewise, the 343- and 419-cm^{-1} Raman bands, which are present in the cobalt(II) rubredoxin spectrum, correspond to stretching vibrations at 313 and 365 cm^{-1} present in iron rubredoxin. The increase in frequency could then be about 40 cm^{-1} for both bands. To our knowledge, our data represent the first set of Raman spectra reflecting Co(II)-S_4 core in a distorted tetrahedral environment ever reported. The same is true for our MCD data, and thus a spectral basis for comparison of model compounds has been established.

In the case of cobalt rubredoxin, the (2Co) species is always obtained upon reconstitution, and our results establish that this species is considerably more stable than the corresponding (2Fe) species. We have obtained no evidence for nonequivalence of metal binding in (2Co)-rubredoxin with respect to metal dissociation, such as has been observed in the 2Fe species. Competitive ligation studies reveal preferential binding of cobalt over iron to the apoenzyme, and this may be a reflection of either kinetic or thermodynamic factors. It is possible that the color difference in reconstitution mixtures before and after exposure to air is an indication that it is actually the reduced states of the metal ions which are initially ligated by the sulfur atoms.

Our data establish that cobalt rubredoxin mediates the reduction of cytochrome *c* in the presence of NADH and the flavoprotein reductase, although the iron enzyme is more efficient in this regard. Since, as judged by difference spectra,

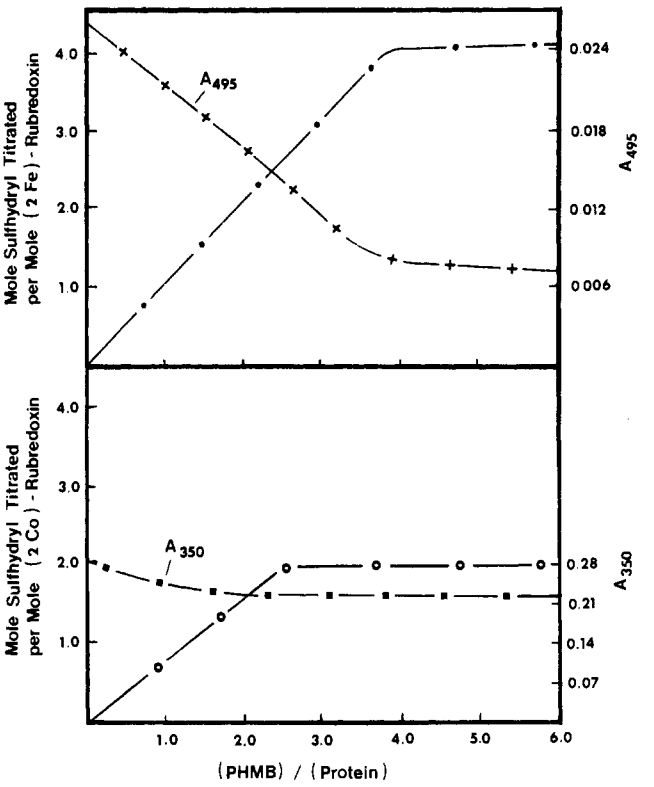

FIGURE 6: Reaction of quantitative amounts of PHMB with (2Fe)- and (2Co)-rubredoxin. The reactions were carried out in 0.1 M Tris-Cl, pH 7.0, at room temperature. Successive stoichiometric additions of the reagent were made to both sample and reference (buffer only) cells. The amount of sulfhydryl reacted was calculated from the increase in absorbance at 250 nm.

cobalt rubredoxin retains the ability to complex with reductase, it is tempting to speculate that a reversible Co(II)–Co(III) interconversion occurs, analogous to the Fe(II)–Fe(III) interconversion in native rubredoxin. According to this hypothesis, the decreased efficiency of the cobalt enzyme could be attributed to an unfavorable potential for facile oxidation to Co(III) (Cotton and Wilkinson, 1972). There are several known Co(III) enzymes, including carbonic anhydrase (Shinar and Navon, 1973), carboxypeptidase A (Kang et al., 1972; Van Wart and Vallee, 1977), alkaline phosphatase (Anderson and Vallee, 1975), and DNA-dependent RNA polymerase (Wu et al., 1977), all of which have been obtained from the Co(II) enzymes by hydrogen peroxide or *m*-chloroperoxybenzoic acid oxidation. It must be emphasized that we have obtained no unequivocal evidence for the existence of a cobalt(III) rubredoxin species, either after H_2O_2 or *m*-chloroperoxybenzoic acid oxidation or upon incubation with cytochrome *c*. A number of spectral changes were observed upon incubation of cobalt rubredoxin with either oxidizing or reducing agents, but the transient nature of these changes makes interpretation tenuous. It should be noted that interaction with reductase could alter the coordination geometry of cobalt sufficiently so as to facilitate reversible oxidation-state changes, and it is possible that such an interaction is being reflected in the d–d region of the difference spectrum.

In our view, it seems reasonable to assume that the two highly reactive sulfhydryl groups of cobalt rubredoxin observed in both Aldrithiol and PHMB titrations are not among those participating in metal binding, since we have found the cobalt atoms to be quite stable toward dissociation, or even toward reaction with 1,10-phenanthroline. The small changes observed in the charge-transfer bands upon modification are much smaller than what would be expected for removal of two sulfur

ligands from cobalt, and they most likely reflect conformational changes upon attachment of the large modification reagents, plus possibly interaction between the cobalt and mercury atoms in the case of PHMB. Thus, our data suggest that direct participation of the "extra" thiol groups at either end of rubredoxin is not essential in electron transport from the reductase. Further studies aimed at defining precisely the role of rubredoxin in the coupling of electron transport to oxygen activation in the epoxidation system of *P. oleovorans* (May, 1976; May and Abbott, 1972; 1973; May et al., 1973, 1974a,b, 1975, 1976, 1977) are currently in progress.

Acknowledgments

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