Rubredoxin from *Pseudomonas oleovorans*: Effects of Selective Chemical Modification and Metal Substitution†

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ABSTRACT: *Pseudomonas oleovorans* rubredoxin is one of three protein components of an oxygenase system previously shown to catalyze terminal methyl group hydroxylation of alkanes and fatty acids and epoxidation of terminal olefins. *P. oleovorans* rubredoxin differs from those of the anaerobes in that it contains one extra sulfhydryl group near each of the two metal binding sites located at either end of the molecule. In order to ascertain whether extra sulfhydryls are essential to activity, they have been subjected to selective chemical modification both in intact rubredoxin and in its C-terminal CNBr fragment. In the case of intact rubredoxin, the native iron atoms were replaced with cobalt to give rubredoxin containing two atoms of cobalt per protein molecule. The relative stability of cobalt binding allowed selective chemical modification with iodoacetamide under conditions which leave the ligation environment of cobalt intact. The modified protein was characterized on the basis of sulfhydryl titration data, special properties, quantitative measurements of modification stoichiometry with iodo[1-^{14}C]acetamide, and the demonstration of comparable radioactivity in N- and C-terminal CNBr fragments. Removal of cobalt from this modified rubredoxin and restoration of iron yielded rubredoxin containing iron at the metal binding site but with the "extra" sulfhydryl groups chemically blocked, although some residual cobalt was not removable. In the case of the rubredoxin C-peptide, the single extra sulfhydryl was modified with methyl methanethiosulfonate, and the modified peptide was fully characterized by spectral and titration data. Activity assays demonstrated that while chemical modification reduced epoxidative activity, both modified rubredoxin and modified C-peptide were still active. As expected, modification with methyl methanethiosulfonate, which introduces a less bulky moiety, caused a significantly smaller activity loss. Chemical modification had little effect on the abilities of the rubredoxins to transfer electrons to cytochrome *c*. Therefore, we conclude that the extra sulfhydryls of *P. oleovorans* rubredoxin are not essential for the activity of this protein in supporting enzymatic oxygenation.

The enzyme system from *Pseudomonas oleovorans* that catalyzes terminal methyl group hydroxylation of alkanes and fatty acids was first shown by Coon and co-workers to contain three protein components: rubredoxin, a flavoprotein reductase, and a non-heme iron containing monooxygenase (Lode & Coon, 1971; McKenna & Coon, 1970; Reuttinger et al., 1974). In previous work we demonstrated that this enzyme system very readily converts terminal olefins to the corresponding 1,2-oxides, and we have examined the specificity, stereochemistry, and mechanism of these enzymatic oxygenations (May, 1979; May & Abbot, 1972, 1973; May & Kuo, 1977, 1978; May & Schwartz, 1974; May et al., 1973, 1974, 1975, 1976, 1977). The weight of evidence now strongly indicates that both epoxidation and hydroxylation occur at the same active site on the "epoxidase/hydroxylase" component, although the lack of a strong functional chromophore has hampered dissection of reaction steps at the molecular level as has been done for P-450- and flavin-containing oxygenases.

P. oleovorans rubredoxin differs from those of the anaerobes in that it contains two metal binding sites, and data with the cyanogen bromide cleaved enzyme support the conclusion that each binding site is composed of cysteine residues from only one end of the molecule (Lode & Coon, 1971). It has been demonstrated that iron bound in the N-terminal site is exceedingly labile (Lode & Coon, 1971), and in fact, rubredoxin is always isolated as the species containing only one iron, which is bound in the C-terminal site. Although atomic absorption analysis indicates that reconstitution of aporubredoxin with 2 equiv of Fe does give (2Fe)–rubredoxin as the immediate product (May & Kuo, 1978), the N-terminal iron is easily lost in solution at room temperature.

The sequence data on rubredoxin (Benson et al., 1971) establish that a cluster of five sulfhydryl groups exists near each terminus, and since the metal ions are clearly tetrahedrally sulfur ligated, a single uncoordinated sulfhydryl group is presumably available near each metal binding site, or alternatively, a diterminal disulfide exists. Extra sulfhydryl groups are not present in rubredoxins from anaerobic bacteria such as *P. elsdenii* (Bachmayer et al., 1968a,b), *Desulfovibro gigas* (Bruschi, 1976b), *Clostridium pasteuranium* (Watenpaugh et al., 1973), *Micrococcus aerogenes* (Bachmayer et al., 1968b), and *D. vulgaris* (Bruschi, 1976a). It is striking that although anaerobic rubredoxins do accept electrons from the *P. oleovorans* reductase and have metal binding sites and redox potentials analogous to those of *P. oleovorans* rubredoxin, the anaerobic rubredoxins are unable to support hydroxylation in the *P. oleovorans* system. It has thus been an important experimental goal to establish whether the extra sulfhydryl groups of *P. oleovorans* rubredoxin play a direct role in the function of this protein.

We have previously reported on the preparation of cobalt rubredoxin in which both metal binding sites of *P. oleovorans* rubredoxin were occupied by cobalt atoms (May & Kuo, 1978). The spectral properties and metal content of cobalt rubredoxin establish the presence of two cobalt(II) atoms in rubredoxin-type binding sites. In contrast to iron rubredoxin, where the lability of the N-terminal binding site precludes selective modification of two sulfhydryl groups per protein molecule (Lode & Coon, 1971; May & Kuo, 1978), our preliminary studies with cobalt rubredoxin indicated that the relative stability of cobalt binding should allow selective chemical modification of the two extra sulfhydryls. We report

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herein on the selective chemical modification of the extra sulfhydryl groups of both *P. oleovorans* rubredoxin and its Fe-C-peptide and on the effects of modification on the activities of these species.

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) was from Aldrich; methyl methanethiosulfonate (MMTS),[1] *p*-(hydroxymercuri)benzoate (pHMB), and ferredoxin–$NADP^+$ reductase were 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. All other materials were obtained, synthesized or purified as previously described (May & Kuo, 1971).

For reconstitution experiments, all buffer solutions used were pretreated either by extracting with dithizone (diphenylthiocarbazone; Fisher) in CCl_4 or by the following procedure: Chelex-100, which had been treated sequentially with 1 M NaOH, water and 10 mM Na_4EDTA, 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. New plastic receptacles, which had been rinsed with distilled, deionized water, were used whenever possible. All glassware was soaked overnight in 2 M HNO_3 followed by both 10 mM EDTA and distilled, deionized water rinses. Highly purified chemicals were used, being transferred either by a Teflon spatula or disposable plastic pipets.

Rubredoxin was isolated from octane-grown cells of *P. oleovorans* according to procedures which have been previously described (May & Kuo, 1977, 1978) except that the cells were lysed by using hypotonic shock rather than sonication, which increased the yield of enzyme obtained by 5–10-fold. The purified protein exhibited normal spectral properties, and purity was established by the criteria we have described elsewhere (May & Kuo, 1978).

P. oleovorans epoxidase/hydroxylase was purified from cell membranes. The thawed membranes were washed for 1 h with 20 mM Tris-HCl, pH 7.4 (1 g of membranes/5 mL of buffer), and pelleted by centrifugation (30 min at 16000*g*). The pellet was suspended in 20 mM Tris-HCl, 2 mM EDTA, 2 mM dithionite, and 0.1% deoxycholate, pH 7.4, buffer (1 g of membranes/3 mL of buffer) and stirred at 4 °C for 2 h. The suspension was sonicated with a Branson sonifier cell disruptor for 3 min at 75% output in blasts of 1 min each. The sonicated material was diluted by 50% with cold buffer (same as for sonification) and after 20 min was centrifuged (1 h at 20000*g*). The supernatant was subjected to ammonium sulfate precipitation, and the 30–40% pellet was kept and resuspended in minimal buffer (20 mM Tris-HCl, 20% glycerol, 0.1% deoxycholate, pH 7.4). The suspension was centrifuged (30 min at 20000*g*), and the supernatant was either frozen or subjected to the next purification step. The material was then applied to a Bio-Gel A-15 column (2.5 cm × 40 cm) and eluted at a flow rate of 10 mL/h. Active fractions were pooled and concentrated in an Amicon cell by using a YM-10 membrane. The epoxidase/hydroxylase could at this point be frozen without loss of activity. The purified enzyme exhibited a single band upon disc gel electrophoresis, but a faint minor component appeared in SDS gel electrophoresis.

Epoxidative activity assays of native metal substituted and chemically modified rubredoxins were carried out by using the quantitative gas chromatographic procedures we have described previously (May & Abbot, 1973). Typically, epoxidase isolated by the above procedure exhibited an activity of 390 mg of 7,8-epoxy-1-octene formed per mg of protein, in the standard 1.2-mL reaction mixture containing saturating concentrations of rubredoxin and reductase. Cytochrome *c* reduction activity was monitored spectrophotometrically as described previously (May & Kuo, 1978). All UV–vis spectra were obtained on either an Aminco DW-2 or Beckman ACTA MVI spectrophotometer at 25 °C.

Preparation of aporubredoxin and reconstitution with either iron or cobalt was performed by using our previously described procedures (May & Kuo, 1978). Modification of cobalt rubredoxin with iodoacetamide was accomplished as follows. Freshly prepared Co-Rd suspended in 0.05 M Tris-HCl, pH 7.3, was adjusted to pH 8.0 by the addition of 1.0 M Tris base and flushed with Ar for 30 min at room temperature with gentle stirring. The vessel was covered with aluminum foil (to prevent the oxidation of released iodide to iodine), and α-iodoacetamide in 1 M NaOH was added, dropwise and anaerobically, until a 2.5:1 molar ratio of iodoacetamide: protein was obtained. After 30 min in the dark under Ar, at room temperature, the material was cooled in ice for 5 min and loaded onto a Sephadex G-75 column (2.5 × 80 cm), which had been equilibrated with 0.05 M Tris-HCl, pH 7.3, and covered with aluminum foil. The sample was eluted with 0.05 M Tris-HCl, pH 7.3. The rubredoxin fractions were combined and stored at −70 °C.

Cobalt-substituted, modified rubredoxin (Co-Mod-Rd) was cleaved by cyanogen bromide in 70% formic acid at room temperature by the general procedures of Steers et al. (1965). Freshly prepared Co-Mod-Rd in 0.05 M Tris-HCl, pH 7.3, was lyophilized and the lyophilysate was dissolved in 70% formic acid (which had been flushed with Ar for 1 h) to a concentration of ~3 mg/mL. The solution was flushed with argon for 15 min and a 50-fold molar excess of cyanogen bromide (dissolved in 0.5 mL of 70% formic acid) was added under an Ar atmosphere. The vessel was covered with aluminum foil and allowed to stand for 24 h at room temperature, under Ar. The reaction mixture was then diluted with 10 volumes of water and lyophilized. The lyophilysate was dissolved in minimal, cold 10% acetic acid and applied to a Sephadex G-75 column (2.5 × 120 cm) which had been equilibrated with 10% acetic acid. The sample was eluted with 10% acetic acid, and the fractions corresponding to three protein peaks were combined separately. Protein fractions 1 and 3 were lyophilized after spectral analysis while fraction 2 was concentrated in an Amicon cell (YM10 membrane), reapplied to the Sephadex G-75 column, and eluted with 10% acetic acid. The appropriate fraction from this column was lyophilized. Likewise, Fe–C-peptide was prepared by the reconstitution of apo-C-peptide, a fragment of CNBr cleavage of aporubredoxin which had been pretreated with 2-

[1] Abbreviations: MMTS, methyl methanethiosulfonate; pHMB, *p*-(hydroxymercuri)benzoate; Aldrithiol-4, 4,4′-dithiodipyridine; Rd, rubredoxin; Co-Rd, cobalt-substituted rubredoxin; Co-Mod-Rd, Co-Rd after iodoacetamide modification; Fe-(Co-Mod)-Rd, Co-Mod-Rd after restoration of iron; Fe-(Co)-Rd, Co-Rd after restoration of iron; Fe-C-peptide, C-terminal CNBr fragment of Rub, after restoration of Fe; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

hydroxyethyl disulfide at pH 9.3 (Lode & Coon, 1971; Steers et al., 1965).

The titration of *total* sulfhydryls in Fe–C-peptide by Aldrithiol-4 was performed after its denaturation by the presence of 10 μL of glacial acetic acid and 1 mM EDTA, followed by the addition of 0.2 mL of 1 M Tris base. MMTS was used to modify the single "extra" sulfhydryl in Fe-C-peptide. For quantitation purposes, MMTS stock solution was standarized as follows. A solution of 2-mercaptoethanol was first standarized by Aldrithiol and was then used to titrate an aliquot of the MMTS stock solution. Back-titration with Aldrithiol-4 then allowed calibration of the actual concentration of the MMTS stock solution. Metal analyses were carried out on a Perkin-Elmer Model 5000 atomic-absorption instrument with an HGA 500 graphite furnace and an AS 40 autosampler. Amino acid analyses were carried out by Dr. James Travis at the University of Georgia on a Beckman 119CL amino acid analyzer. Sulfhydryl titrations with Aldrithiol-4 and PHMB were carried out spectrophotometrically as we have previously described (May & Kuo, 1978).

Results

A unique feature of *P. oleovorans* rubredoxin which distinguishes it from rubredoxins of anaerobes is the fact that it contains two iron binding sites, each composed of cysteine residues from only one end of the molecule (Lode & Coon, 1971). In previous work from this laboratory we have established conditions for removal of iron and for reconstitution with cobalt salts which afford Co-Rd containing two Co(II) atoms per rubredoxin molecule, each one of which is in a tetrahedrally cysteine-coordinated binding site (May & Kuo, 1978). As we have discussed in detail previously, the spectral properties of Co-Rd as well as its metal content, are fully consistent with the presence of two Co(II) atoms in rubredoxin-type binding sites. Notable are the 350-nm thiolate to Co(II) charge-transfer band and the d–d bands at 620, 685, and 748 nm expected for the distorted tetrahedral high-spin Co(II) system. As previously discussed, the extinction coefficients indicate that a total of eight Cys-S groups are involved in coordination of the two cobalt atoms (May & Kuo, 1978).

Chemical Modification of the Extra Sulfhydryls of P. oleovorans Rubredoxin. Our own previous work (May & Kuo, 1978) and that of Lode and Coon (1971) have established that the lability of the second iron in (2Fe)–rubredoxin precludes selective modification of the two extra sulfhydryls in this species. In contrast, our preliminary work has established that due to the relative stability of cobalt binding to both metal binding sites, two highly reactive sulfhydryls could be selectively titrated in Co-Rd (May & Kuo, 1978). We have now successfully modified these two reactive sulfhydryls of Co-Rd with iodoacetamide under the conditions outlined under Materials and Methods to produce Co-Mod-Rd. The following data establish that two sulfhydryls per Co-Rd molecule have indeed been modified in Co-Mod-Rd, that these are *not* among those participating in metal binding but are extra sulfhydryls, and that the ligation environment of cobalt in Co-Mod-Rd is intact.

(1) Reaction of Co-Rd with iodo[1-^{14}C]acetamide (28.6 Ci/mol), followed by Sephadex G-25 chromatography, gave Co-Mod-Rd cleanly with a protein content of 4.853×10^{-8} mol/mL and radioactivity of 2.84×10^{-6} Ci/mL. Thus, 2.05 ± 0.19 sulfhydryls per protein molecule had been alkylated with iodoacetamide.

(2) Since each of the two binding sites of *P. oleovorans* rubredoxin is composed of cysteines from only one end of the molecule (Lode & Coon, 1971) and five cysteines are located near each terminus (Benson et al., 1971), one extra sulfhydryl group must be located at each end of the molecule. Inspection of the sequence data indicates that these are likely to be cysteines-40 and -157. Thus, if iodoacetamide modification has successfully alkylated the extra thiols, CNBr cleavage of Co-Mod-Rd at the single Met, located at position 50 between the cysteine clusters, should result in C- and N-terminal fragments with equal contents of the iodoacetamide functionality. On the other hand, if cobalt substitution has not succeeded in blocking the N-terminal labile Fe site, iodoacetamide alkylation followed by CNBr cleavage should result in a preponderance of alkylation in the N-terminal fragment, as is the case for native Fe-Rd (Lode & Coon, 1971; May & Kuo, 1978). CNBr cleavage of [^{14}C](Co-Mod-Rd) followed by fragment separation of G-75 Sephadex (Materials and Methods) yielded the N- and C-terminal peptides. Each fragment was identified by amino acid analysis and comparison of the data with the known sequence of rubredoxin and the differing composition of the two fragments (Benson et al., 1971; Lode & Coon, 1971). The specific radioactivities found for the C- and N-terminal fragments were comparable, 2.5×10^{-8} and 2.0×10^{-8} Ci/nmol, respectively.

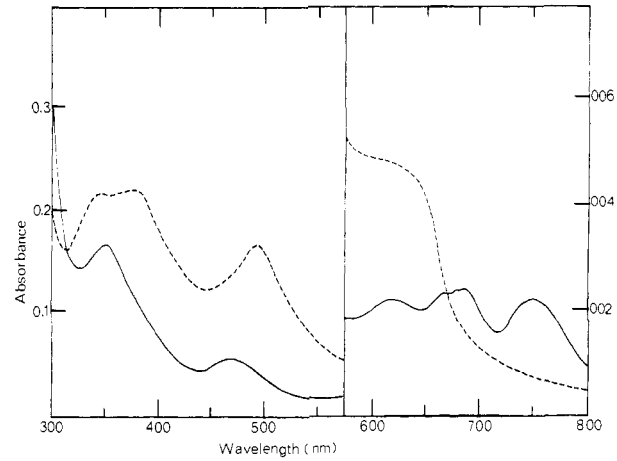

FIGURE 1: Absorption spectra of Co-Mod-Rd (—) and reconstituted Fe-(Co-Mod)-Rd (- - -). Co-Mod-Rd was prepared by modification of Co-Rd with iodoacetamide. Fe-(Co-Mod)-Rd was prepared from Co-Mod-Rd by removal of cobalt and reconstitution with iron.

(3) Figure 1 shows the absorption spectrum obtained for Co-Mod-Rd. As expected, the spectral features of Co-Rd are essentially unchanged after modification; however, modification of metal-ligating sulfhydryls would have resulted in large changes in the intensity of the charge-transfer bands and in major distortions in the d–d bands. Atomic absorption spectrometry of Co-Mod-Rd revealed the presence of 2.08 cobalt atoms per molecule, consistent with the intensity of the 350-nm charge-transfer band which exhibits an extinction coefficient of approximately 1000/Co–S-Cys bond in both proteins and model systems (May & Kuo, 1978). Thus, we conclude that both metal content and ligation environment are unchanged after iodoacetamide alkylation.

(4) Our preliminary work with Aldrithiol-4 has established the presence of two highly reactive sulfhydryls in Co-Rd, both capable of being selectively modified with PHMB. Comparative titrations with native rubredoxin, as well as spectral analysis, strongly indicated that these are, in fact, the extra sulfhydryls not involved in metal binding (May & Kuo, 1978). Thus, Co-Mod-Rd prepared by iodoacetamide alkylation should exhibit loss of these two reactive sulfhydryls in Aldrithiol-4 titrations. Figure 2 illustrates sulfhydryl titration data for Co-Rd and Co-Mod-Rd. It is immediately evident that the two rapidly reacting sulfhydryls of Co-Rd are absent

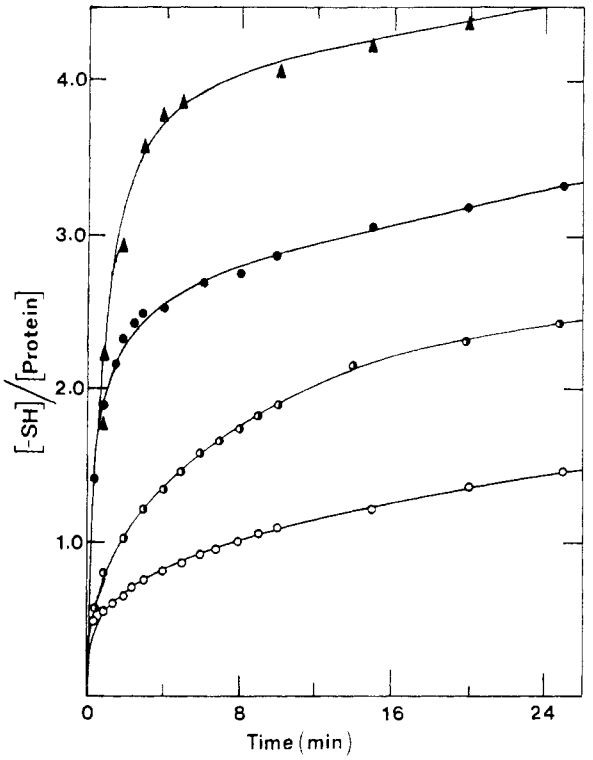

FIGURE 2: Sulfhydryl titrations of rubredoxin species with Aldrithiol-4. Typically, 0.1 mg of the protein sample was suspended in 1.0 mL of 0.1 M Tris-HCl buffer, pH 7.3, a 10:1 molar ratio of Aldrithiol-4:protein was added, and the change in absorbance at 324 nm was recorded with time. The molarity of -SH groups titrated was calculated by using ϵ_{324} = 19 800 M^{-1} cm^{-1}. (▲) Fe-Rd; (●) Co-Rd; (○) Co-Mod-Rd; (◑) Fe-(Co-Mod)-Rd.

in Co-Mod-Rd. Loss of reactive sulfhydryls is also clearly evident in the Fe-(Co-Mod)-Rd species prepared from Co-Mod-Rd by metal removal and restoration of iron, as discussed below.

Removal of Cobalt and Restoration of Iron. Conversion of Co-Mod-Rd back to the iron form was carried out by using our previously described procedure (May & Kuo, 1978) in order to obtain Fe-Rd with its extra sulfhydryls cleanly alkylated. The absorption spectrum obtained for this species (Figure 1) closely resembles published spectra for Fe-Rd, exhibiting the highly characteristic 497- and 360-nm bands (Lode & Coon, 1971; May & Kuo, 1977, 1978). However, the shoulder at 350 nm is more pronounced than in native rubredoxin, which suggested to us the presence of residual cobalt. Atomic absorption analysis indeed indicated the expected iron content of 1.1–1.2 atoms per molecule but a residual cobalt content of 0.1–0.28 atoms per molecule depending on the particular preparation and the number of trichloroacetic acid precipitation steps during metal replacement. Thus, we designate this iron-restored species as Fe-(Co-Mod)-Rd. Fe-(Co-Mod)-Rd titrations with Aldrithiol-4 confirmed the continued absence of reactive sulfhydryls, although as we have previously discussed (May & Kuo, 1978), sulfhydryl titrations of iron rubredoxin species do not show the clean breaks in reactivity characteristic of pure cobalt species.

Activities of Modified and Metal-Replaced Rubredoxins. The various metal-substituted and chemically modified rubredoxins were assayed for their activities both in supporting the rubredoxin-dependent reduction of cytochrome *c* (Lode & Coon, 1971) and in supporting epoxidation by the reconstituted *P. oleovorans* epoxidation/hydroxylation system. As shown in Table I, apo-Rd prepared from either Fe-Rd or Co-Mod-Rd was essentially inactive, the slight activity of the

Table I: Electron-Transfer Activity to Cytochrome *c* of Modified and Metal-Replaced Rubredoxins

Rd species	nmol of Rd	activity (A_{550}/min)[a]
Fe-Rd	0.07	0.20
	0.18	0.57
	0.35	0.91
Co-Rd	0.07	0.16
	0.17	0.29
	0.33	0.55
	0.42	0.65
apo-Rd	0.27	0.00
	0.53	0.01
Fe-(Co-Mod)-Rd	0.11	0.24
	0.21	0.40
	0.43	0.90
Co-Mod-Rd	0.10	0.07
	0.25	0.42
	0.50	0.62
apo/Co-Mod-Rd	0.16	0.03
	0.32	0.03

[a] Reaction mixtures containing 50 μmol of Tris-HCl, pH 7.3, varying amounts of rubredoxin (0–600 pmol), 0.3 mg of cytochrome *c*, and 10.5 μg of spinach ferredoxin-NADP$^+$ reductase were preincubated at room temperature for 5 min, and the reduction of cytochrome *c* was initiated by the addition of 0.25 mg of NADPH. The increase in absorbance at 550 nm was recorded, and the data reported are corrected for the small reduction that occurs without the presence of rubredoxin.

latter being undoubtedly due to residual cobalt which is very difficult to remove as indicated above. On the other hand, all other rubredoxin species were active, clearly indicating that neither cobalt substitution nor sulfhydryl modification abolishes the cytochrome *c* activity of rubredoxin. Turning to activities in supporting epoxidation, activities of Fe-Rd, Fe-(Co-Mod)-Rd and Co-Rd were examined by using our previously described procedure (May & Abbott, 1973), over the concentration range 0–10 nmol of rubredoxin in a total volume of 1.00 mL. It was immediately evident that cobalt substitution completely abolishes epoxidative activity. On the other hand, selective modification of the extra sulfhydryl groups of rubredoxin results in Fe-(Co-Mod)-Rd which retains approximately 25% of the activity of Fe-Rd. As a control, Fe-(Co)-Rd was prepared by a procedure identical with that used for the preparation of Fe-(Co-Mod)-Rd and fully characterized. This species, where cobalt was removed and iron restored but without chemical modification, exhibited activity in supporting epoxidation comparable to that of native Fe-Rd. It is important to note that the presence of residual cobalt does not affect conclusions drawn from these activity comparisons since cobalt-containing sites are inactive in supporting epoxidation and all activities are compared on the basis of iron content in the rubredoxin species.

Chemical Modification of the Extra Sulfhydryl of the Fe-C-Peptide Fragment. It is evident from the above that while cobalt substitution was necessary in order to allow selective modification of only the extra sulfhydryls of intact rubredoxin, it was not possible to fully remove the cobalt and restore the iron. We therefore wished to focus on the C-terminal CNBr fragment of rubredoxin, since Lode & Coon (1971) had previously reported that this fragment retains approximately 30% of the activity of intact rubredoxin in supporting hydroxylation. We anticipated that iron ligation in the Fe-C-peptide would be sufficiently stable so as to allow selective modification of the single extra sulfhydryl without resort to the intermediacy of a cobalt-substituted species. If so, we would be able to use MMTS instead of iodoacetamide as the sulfhydryl modification reagent, which would result in

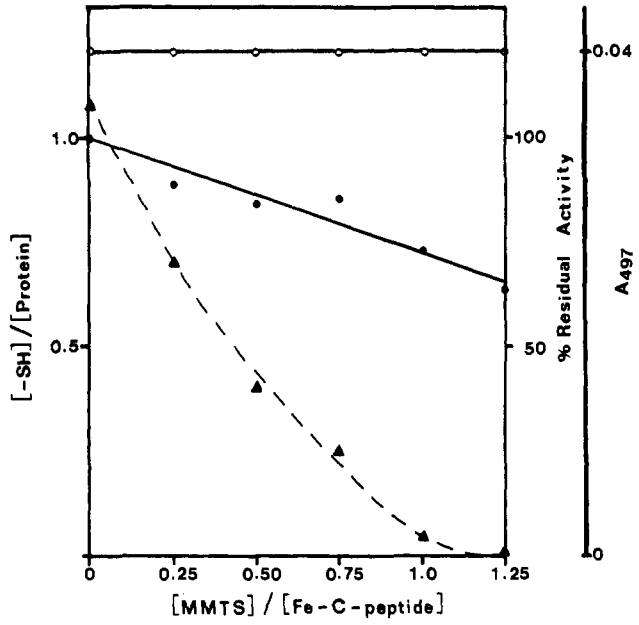

FIGURE 3: Titration of the Fe-C-peptides with methyl methanethiosulfonate (MMTS). Aliquots of Fe-C-peptide, each containing 6.0 nmol in 0.9 mL of Tris buffer, were modified with various amounts of 1.4 mM MMTS at pH 7.4. Samples were incubated for 30 min in an ice bath, followed by back-titration with 10 nmol of Aldrithiol-4 at room temperature. The concentration of MMTS had been previously standardized by 2-mercaptoethanol and Aldrithiol-4, as described under Materials and Methods. The number of residual sulfhydryl groups after modification is indicated by (▲). The absorbance of the Fe–C-peptide at 497 nm (○) did not change throughout the modification and titration procedure. The activity plot (●) was normalized on the basis of 100% activity by unmodified Fe-C-peptides. The concentration of Fe-C-peptide species for GC assay was always 6.0 nmol. An activity of 100% corresponds to 82 ng/mL epoxide product.

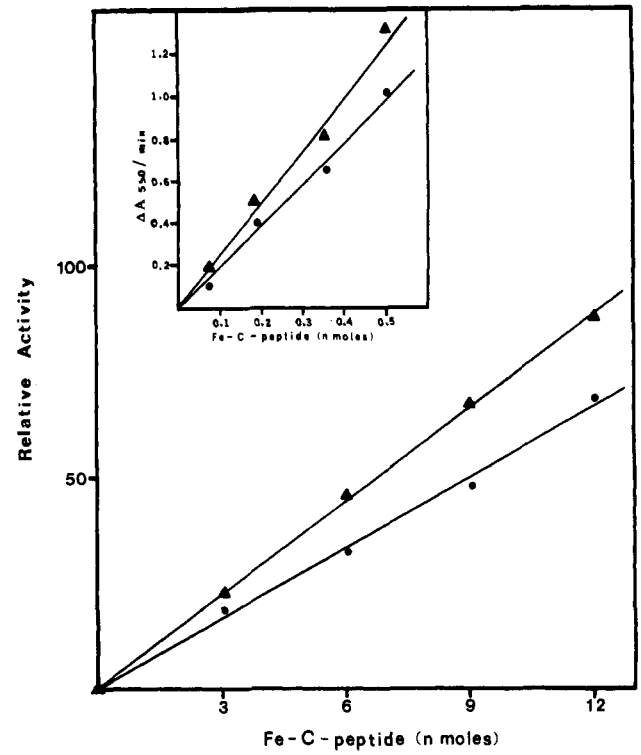

FIGURE 4: Activity of Fe-C-peptides species with epoxidase. (▲) Fe-C-peptide; (●) Fe-C-peptides that have been modified with 1.5 molar excess of MMTS. (Insert) Electron transport activity to cytochrome *c* of native (▲) and modified (●) Fe-C-peptide. Conditions are the same as in Table I.

incorporation of the much less bulky $-SCH_3$ group, thereby reducing steric interference with epoxidation activity. The use of MMTS with intact rubredoxin was precluded since the modification would be reversed under the condition used for removal of cobalt and restoration of iron.

The Fe-C-peptide fragment (M_r 13 000) was obtained by CNBr cleavage of aporubredoxin after protection of the cysteines with 2-hydroxyethyl disulfide (Lode & Coon, 1971; Steers, 1965). The apopeptide was isolated by Sephadex G-75 chromatography and reconstituted with a stoichiometric amount of iron. The spectral properties of the Fe-C-peptide were the same as those described by Lode & Coon (1971) with an A_{280}:A_{497} ratio of 4.3. After denaturation of the Fe-C-peptide by incubation for 10 min in glacial acetic acid containing 1 mM EDTA, a total of five sulfhydryls were titratable with Aldrithiol-4. On the other hand, only one sulfhydryl was detectable by Aldrithiol-4 titration of the undenatured native Fe-C-peptide. After the Fe-C-peptide had been incubated with 1 equiv of MMTS for 30 min at 0 °C, no Aldrithiol-4-titratable sulfhydryls could be detected. These results confirm our expectation that the single extra sulfhydryl of the Fe-C-peptide is available for chemical modification.

The results of MMTS titrations of the Fe-C peptide are shown in Figure 3, where residual Aldrithiol-4-titratable sulfhydryls, A_{497}, and residual activity in supporting epoxidation are plotted. It is evident that as increasing amounts of MMTS are added, modification of the single Aldrithiol-4-titratable sulfhydryl occurs. However, as expected, no change in the characteristic 497-nm charge-transfer band is evident as the extra sulfhydryl becomes modified, the activity of the Fe-C-peptide in supporting epoxidation decreases by 30% at the single concentration of rubredoxin tested (6.0 nmol; see legend to Figure 3).

The concentration dependencies of the activities of native and modified Fe-C-peptide in supporting epoxidation are shown in Figure 4. It is evident that for both species activity is linearly dependent on concentration in the range tested. Although modification has reduced the activity somewhat, the modified Fe-C-peptide is clearly catalytically competent throughout the concentration range. Similarly, as shown in the insert, modification results in only a small decrease in the cytochrome *c* reducing activity of the Fe-C-peptide.

Discussion

Taken together, the data reported in this paper establish that substitution of the iron of *P. oleovorans* rubredoxin with Co(II) allows selective modification of the nonmetal-binding sulfhydryls of this protein with α-iodoacetamide. Support for this conclusion comes from the Aldrithiol titration data, the spectral properties of the modified rubredoxins, quantitative measurements of modification stoichiometry with iodo[1-^{14}C]-acetamide, and the demonstration of comparable radioactivity in the N- and C-terminal CNBr fragments. Such selective modification of the extra thiols has not been possible with native Fe-Rd, due to the extreme lability of the iron in the N-terminal binding site. Modification of these extra sulfhydryls, which are unique to *P. oleovorans* rubredoxin, has enabled us to examine their possible involvement in the activity of this protein as a component of the *P. oleovorans* epoxidation/hydroxylation system.

Both Co-Rd and Co-Mod-Rd were found to actively reduce cytochrome *c* in the presence of spinach ferredoxin–$NADP^+$ reductase, NADPH, and molecular oxygen. Previously we reported a substantial difference in cytochrome *c* reduction activities between Rd and Co-Rd in the concentration range 20–250 nmol (May & Kuo, 1978), which is 100–1000 times the concentration range in Table I. However, it is clear that

Co-Rd has good activity under both sets of conditions. Furthermore, Co-Mod-Rd is virtually as active as Co-Rd, and similarly, restoration of iron into modified rubredoxin gives Fe-(Co-Mod)-Rd with activity comparable to that of the native enzyme, thus clearly indicating that the two extra sulfhydryls are not directly involved in this electron-transfer process. With regard to the cobalt-substituted rubredoxin species, it is possible that the detailed mechanism of cytochrome *c* reduction differs from that operative for the iron-containing species. However, this possibility does not affect our conclusions regarding the consequences of chemical miodification. In this regard, we note that the rate of cytochrome *c* reduction with Fe-Rd remains constant until all cytochrome *c* is consumed but, under identical conditions, the rate of reduction with Co-Rd and Co-Mod-Rd follows a hyperbolic curve.

Turning to oxygenase activity, both Co-Rd and Co-Mod-Rd are unable to support epoxidation in the reconstituted *P. oleovorans* system over a wide range of concentrations. Apparently either the cobalt-substituted rubredoxin is unable to interact with the epoxidase/hydroxylase or the reduction potential of the cobalt atom is too high and does not permit the flow of electrons to the monooxygenase. With regard to the first possibility, we have previously established that Co-Rd exhibits spectral properties indicative of normal tetrahedral ligation of the metal (May & Kuo, 1978), but accessibility to the epoxidase/hydroxylase may be restricted upon metal substitution. Irrespective of these considerations, it is clear that when iron is restored to Co-Rd, the resulting Fe-(Co)-Rd is fully active in supporting epoxidation. Thus, the process of removing the native iron does not alter the protein structure so as to render rubredoxin catalytically incompetent with respect to enzymatic oxygenation.

The major goal of this work was to selectively modify the extra sulfhydryls of rubredoxin in order to examine their role in the functioning of this protein. Our results with native rubredoxin show that modification reduced epoxidative activity considerably but that the modified protein was still active. In view of the complications attributable to lability of the N-terminal iron binding site, we also chose to examine the effect of modification on the activity of the Fe-C-peptide. For the Fe-C-peptide, it was possible to utilize MMTS as the modification agent, thereby attaching a much less bulky moiety to the extra sulfhydryl. The resulting modified Fe-C-peptide exhibited a significantly smaller loss in activity than had been the case of the Fe-(Co-Mod)-Rd. Thus, we conclude that the extra sulfhydryls of rubredoxin are not essential for the activity of this protein. The reduced activities of the modified Rd and Fe-C-peptide species may be due to a number of factors such as steric interference with interaction with epoxidase, conformational alterations, and the like. The fact that MMTS modification was less detrimental to activity than alkylation with iodoacetamide may reflect differences in steric bulk, hydrophobicity, or hydrogen-bonding ability of the blocking groups incorporated by these two reagents. Similar considerations have been suggested by Smith et al. (1975) to explain the differing effects of sulfhydryl modification reagents on the activity of creatine kinase.

In view of our conclusion that the extra sulfhydryls are not directly involved in catalysis, the inability of the anaerobic rubredoxins to support oxygenation despite their normal redox potentials and their abilities to accept electrons from the *P. oleovorans* reductase (Lode & Coon, 1971) remain enigmatic. One possibility is that all or part of the middle portion of the *P. oleovorans* rubredoxin molecule [i.e., the region between residues 50 and 107 (Benson et al., 1971)] is necessary for interaction with the epoxidase. This intermediate region is lacking in the anaerobic rubredoxins but is present in the Fe-C-peptide which is capable of supporting epoxidation and hydroxylation. Further studies on the interaction of rubredoxin and epoxidase will obviously be necessary in order to understand the details of protein interaction and oxygen activation in this system.

Registry No. Methyl methanethiosulfate, 2949-92-0; iodoacetamide, 144-48-9; 4,4′-dithiodipyridine, 2645-22-9; epoxidase, 9054-91-5; cytochrome *c*, 9007-43-6.

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