Biochimica et Biophysica Acta 851 (1986) 157–165
Elsevier

BBA 42092

Methionine-80-sulfoxide cytochrome *c*: preparation, purification and electron-transfer capabilities

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(Received April 4th, 1986)
(Revised manuscript received May 21st, 1986)

Key words: Cytochrome *c*; Chemical modification; Electron transfer; Sulfoxide

In order to explore the electron-transferring properties of methionine-80-sulfoxide cytochrome *c*, the pure, chromatographically homogeneous methionine-80-sulfoxide cytochrome *c* was prepared. A previously published procedure (Ivanetich, K.M., Bradshaw, J.J. and Kaminsky, L.S. (1976) Biochemistry 15, 1144–1153) was found to produce a mixture of products. In the pure derivative, visible spectroscopy indicates that the 695 nm band indicative of the Met-80-Fe coordination is missing, amino acid analysis indicates that only one methionine is modified to the sulfoxide, and the $E^{0'}$ is found to be 240 mV vs. N.H.E. For succinate cytochrome *c* reductase activity, the K_m for modified cytochrome was about one-ninth that of the native protein, while the maximum turnover number of the reductase with the modified protein was only about 54% of that with native protein. In contrast, the activity with cytochrome oxidase measured polarographically using ascorbate and TMPD under two different buffer/pH conditions, gave K_m values that were very similar for both the native and modified cytochromes *c*, but the maximum turnover numbers of the oxidase with the modified protein were less than 40% of native in either buffer. It is concluded that the Met-80-sulfoxide cytochrome *c* in the reduced form is able to maintain substantially its heme crevice structure and thus maintain K_m values similar to those of native protein. However, the low maximum turnover numbers for oxidase activity with the modified protein in the reduced state indicate that electron transfer itself has been significantly decreased, probably because the parity of acid/base and electrostatic interactions of Met-80 sulfur with the Fe in the two redox states has been disrupted.

Introduction

Cytochrome *c* is a member of the electron-transport chain, transferring electrons from Cyt c_1 to Cyt aa_3. After a complex is formed between the Cyt *c* and Cyt aa_3, intramolecular (intracomplex) electron transfer from Cyt *c* to Cyt *a* occurs. In an intramolecular electron transfer between two metalloproteins such as these, one of the key factors that determines the energetics and thus the rate of the electron transfer is the extent of changes in ligand–metal bond distances that occur when the Cyt *c* interconverts between the oxidized and reduced state. One important but functionally poorly understood ligand–meal bond of the cytochromes *c* is the ubiquitous axial coordination of the methionine side chain sulfur (Met 80) to the heme iron, with the sixth axial ligand being histi-

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Abbreviation: Cyt, cytochrome.

dine 18. The absorption band at 695 nm has been assigned to the Met 80-Fe coordination [1,2] and has also been used as an indicator of both this coordination bond and as a measure of the conformational integrity of the protein [3]. Although there is evidence that the Met 80-Fe coordination bond is much stronger in the ferro than ferricytochrome *c* [4], work with Cyt *c* models [5] shows that the Fe-S bond is essentially the same length in the two oxidation states. To explain this unexpected result Mashiko et al. [5] note that while the Fe(III)-S bond length would be expected to be longer than than that of Fe(II)-S since the 'hard' Fe(III) and 'soft' thioether are not very compatible, this effect is offset and compensated for by the increased charge attraction of Fe(III) for the sulfur. Additionally, the X-ray crystallographic study of both the oxidized and reduced horse-heart Cyt *c* [6,7] show that for ferricytochrome *c* (which contained two molecules per unit cell) the average Met-80 sulfur-Fe distances are 2.28 Å and 2.26 Å, but is 2.32 Å for the reduced molecule. These small changes in coordination bond lengths result in decreased nuclear motion during the interconversion of the Fe(II) and Fe(III) states.

According to the Frank–Condon principle, these compensating effects should facilitate rapid electron transfer in the native protein. The Frank–Condon principle requires that that the nuclei do not have time to change either their positions or their momenta during the actual (instantaneous) electron transfer [8]. This requires then that all adjustments of nuclear configuration must be made prior to electron transfer. If, as in the case of cytochrome *c*, these adjustments are already made in both redox states, i.e., very similar ligand–metal bond lengths, then there will be fast electron transfer, since little energy will be needed for reorganization of the metal–ligand bonds. In this light it is of considerable interest and importance to understand in more detail the role played by Met 80-Fe coordination bond in the electron transfer mechanism of Cyt *c*. An important approach to this problem is the biological and physicochemical study of the redox properties of the Met-80-sulfoxide Cyt *c*. Unlike all other modifications of the Met 80 which fully disrupt the Fe-S coordination and also disrupt the internal structure of the Cyt *c* [9], the formation of the sulfoxide of methionine-80 represents a small enough modification so that the heme crevice region is only slightly opened [10] and furthermore, NMR experiments show that at least partial S-Fe coordination is maintained in the ferro and probably the ferri state [11]. This latter property of the Met-80-sulfoxide Cyt *c* makes it an excellent derivative to experimentally test the 'hard' and 'soft' sulfur interactions with the Fe (II, III) states discussed above.

In this work we present the preparation and purification of chromatographically homogeneous methionine-80-sulfoxide Cyt *c*. The original preparation of Met-80-sulfoxide Cyt *c* was reported by Folin [12] and a more recent preparation and characterization was reported by Ivanetich et al. [13]. In this latter work the oxidation of the methionine-80 residue was accomplished via methylene blue mediated photooxidation. In pursuing our own studies of Cyt *c* electron-transfer mechanisms, we sought to prepare methionine-80-sulfoxide cytochrome *c* and discovered that the previous preparation by Ivanetich et al. [13] provides a mixture of photooxidized Cyt *c* products and not a single product. Since previous work with 'Met-80-sulfoxide cytochrome *c*' was probably done with a mixture of modified cytochromes *c*, it was important to reexamine the properties of the homogeneous singly modified species.

Experimental Methods

Purification of native horse heart cytochrome *c*. Horse heart cytochrome *c*, type VI was obtained from Sigma (St. Louis, MO) and was purified by chromatography on carboxymethyl cellulose (CMC, Whatman, New Jersey) as described by Brautigan et al. [14]. Typically two grams of Type VI Cyt *c* were dissolved in 5 mM pH 7.5 buffer and applied to a 2.5 cm × 100 cm column of CMC-32 which had been preequilibrated to pH 7.5 with 50 mM phosphate buffer. The major band of chromatographically homogeneous Cyt *c* was either eluted and collected or cut out from the column by extrusion of the column material, followed by elution of the cytochrome at front with 1.0 M NaCl/50 mM phosphate (pH 7.5). The protein was diluted, applied to a small CMC-32 column (pH 7.5, 50 mM phosphate) and con-

centrated by eluting the protein at front with 1.0 M NaCl/50 mM phosphate, pH 7.5; salt was removed by passage of the concentrated Cyt *c* over a 2.5 cm × 60 cm Sephadex G-25 column equilibrated with 0.1 M ammonium bicarbonate, and the eluted protein was then lyophilized.

Preparation of methionine-sulfoxide cytochromes *c*. The mixture of methionine sulfoxide cytochromes *c* were prepared according to the method described by Ivanetich et al. [13]. Throughout this work only highly purified chromatographically homogeneous native Cyt *c* was used in the modification and other experiments. Four 300 W, 120 V tungsten lamps with 12 inch diameter reflectors were placed symmetrically around and 20 cm from the cylindrical water jacketed reaction vessel. The reaction was maintained at 4°C with a K-4/12D circulating constant temperature bath (Brinkman, Westbury, NY). In a typical reaction, 35 mg purified Cyt *c* were dissolved in 1 ml 84% (v/v) acetic acid solution. This protein solution was then placed in the reaction vessel, and 25 ml 84% acetic acid was added along with 1 ml of $2.09 \cdot 10^{-2}$ M Methylene blue (Kodak, Rochester, NY) which also had been dissolved in 84% acetic acid. As soon as the methylene blue was added, the entire reaction was thoroughly stirred by bubbling oxygen into the solution. Pure oxygen was bubbled into the solution from the bottom of the reaction vessel at a moderate rate with a small tipped bubbling tube for the duration of the reaction. The lights were turned on and the reaction was run for 1 h. The reaction mixture was then transferred to a 100 ml round bottom flask and lyophilized to dryness. The methylene blue was separated from the modified proteins by dissolving the lyophilized product in a small volume of 0.1 M ammonium bicarbonate and passing it over a Sephadex G-25 column (5 cm × 60 cm) which had been pre-equilibrated with 0.1 M ammonium bicarbonate. The eluted cytochrome band was then lyophilized, redissolved in double-distilled water, and again, lyophilized.

Purification of methionine-80-sulfoxide cytochromes *c*. Analytical high resolution chromatography of the lyophylized cytochromes *c* was done on a 1.5 cm × 32 cm column of CMC-32 resin, equilibrated to pH 7.5, 50 mM phosphate. Typically, 25 mg of the unpurified product was applied to the column for chromatography. Fractions of 7.5 ml were collected with an Ultra Rack LKB 7000 automatic fraction collector (Bromma, Sweden). The fractions were read at 410 nm on a Cary 16 UV-visible spectrophotometer. Analytical co-chromatography of native Cyt *c* with the photo-oxidized cytochromes was also done under similar conditions.

Preparative scale chromatographic separation and purification of the modified cytochromes was accomplished with a 2 cm × 90 cm column of CMC-32 which, as above, had been equilibrated to pH 7.5, 50 mM phosphate. In addition individual fractions were subjected to linear gradient chromatography on an analytical scale as described earlier; the gradient used was 750 ml 30 mM is phosphate (pH 7.5) vs. 750 ml 60 mM phosphate (pH 7.5). They proved to be homogeneous.

In order to compare our work with previous studies, chromatography was also done on the Amberlite IRC-50 resin (Rohm & Haas, Philadelphia, PA) using the same conditions as described by Ivanetich et al. [13]. Typically a 1.5 cm × 37 cm column of Amberlite IRC-50 was equilibrated to pH 8.7 172 mM (0.34 M Na^+) phosphate buffer. Amberlite IRC-50 AR is the same as Amberlite CG-50 AR in all respects excepting mesh size. The finer mesh of IRC-50 AR would be expected to provide higher chromatographic resolution than the CG-50 resin used by Ivanetich [13]. About 25 mg oxidized Cyt *c* product were applied to the column. The sample was eluted from the column under the conditions described above and the absorption of the fractions was determined at 410 nm.

Isoelectrofocusing experiments. Isoelectrofocusing of the IRC-50 AR eluted reaction product was done with an LKB 810 (110 ml) isoelectrofocusing column. The anode solution consisted 10 ml of 10 mM acetic acid, and the cathode solution consisted of 15 g sorbitol and 5.5 ml of ml of 1 M of sodium hydroxide in 19.3 ml of water. The light gradient solution consisted of 2.7 g sorbitol/37.5 mg lysine (free base)/0.3 ml pH 9–11 Physiolytes (Brinkman, New York)/0.1 ml pH 8–10 Physiolyte/0.15 ml pH 5–7 Physiolytes in 52.3 ml water. The dense gradient solution consisted of 27 g sorbitol/75 mg arginine/2.1 ml pH 9–11 Physio-

lytes in 34.9 ml of water. Equal volumes of the gradient solutions were used. A linear gradient was formed with an LKB gradient mixer and a peristaltic pump was used to apply it. The 5 mg sample was added in the middle of the gradient at the appropriate gradient density. The potential was set at 550 V and then increased to 800 V after 1.5 h. The focusing was continued for another 11 h and maintained at 4°C throughout the experiment. 1-ml fractions were collected as the column was eluted with a peristaltic pump and absorbances were read at 434 nm. The pH of the fractions was determined at 26°C.

Spectroelectrochemical determination of equilibrium formal reduction potentials of the modified cytochromes. The equilibrium redox potentials of the CMC-32 chromatographed cytochromes *c* were determined spectroelectrochemically using an optically transparent thin layer electrochemical cell as described by Heineman et al. [15]. The redox dye mediator used was 2,6-dichlorophenolindophenol, $E^{0\prime}_{7.0} = 227$ mV vs. N.H.E. All formal reduction potentials are reported vs. the N.H.E. In this method, the redox state of the purified cytochrome *c* fractions were established with the optically transparent electrode set at various applied potentials and the ratio of Cyt c_{ox}/Cyt c_{red} determined spectrophotometrically with the Cary 17. Each fraction was evaluated several times.

Removal of polymeric cytochrome. In previous work on chemically modified cytochromes *c* by Feinberg and Brautigan [16], significant polymerization occurred. To verify whether or not this was the case in this work, the CMC-32 purified protein fractions were subjected to Sephadex G-75-80 chromatography. No polymer formation was observed.

Cytochrome *c* oxidase and reductase activities of Met-80 sulfoxide cytochrome *c*. Ascorbate-TMPD-cytochrome *c* oxidase and succinate cytochrome *c* reductase activities were determined with Keilin-Hartree particle preparations and measured polarographically according to the procedures described by Ferguson-Miller et al. [17] and Brautigan et al. [14]. Oxidase and reductase maximal turnover numbers, TN_{max}, and the Michaelis constants, K_m, were determined and compared for their enzymic reaction with native and methionine-80-sulfoxide cytochromes *c*. Velocities were originally measured polarographically as oxygen uptake in nmoles $O_2 \cdot s^{-1}$. These rates were converted to turnover numbers by multiplying by 4 (to give the nanomoles Cyt *c* per s) and dividing by the total nmoles of Cyt aa_3 in the reaction vessel. Cyt *c* oxidase activity was measured in two different pH's and buffers: 25 mM Tris cacodylate (pH 7.9)/0.08 nmol cytochrome aa_3/1.8 ml total volume and 50 mM potassium phosphate (pH 6.5)/0.02 nmol Cyt aa_3/1.8 ml total volume. Reductase activity was measured in 25 mM tris cacodylate (pH 7.9)/0.1 nmol Cyt aa_3/14 mM succinate/1.8 ml total volume.

Results

The crude protein fraction as obtained from methylene blue oxidation as Cyt *c* was subjected to high resolution CMC-32 chromatography as described in Experimental Methods. The results, which were obtained consistently for many experiments (for both the oxidation reactions and chromatographies) are shown in Fig. 1. Three main protein fractions were consistently obtained. The two small fractions preceding peaks 1 through 3 were considered to be significantly altered or denatured protein and were not explored further. Although not shown in Fig. 1, it was consistently observed that no protein fractions were eluted after peaks 3, indicating that all the native protein had reacted during the modification. Also using the similar experimental conditions, fraction 3 (see Fig. 1) was co-chromatographed with native cyto-

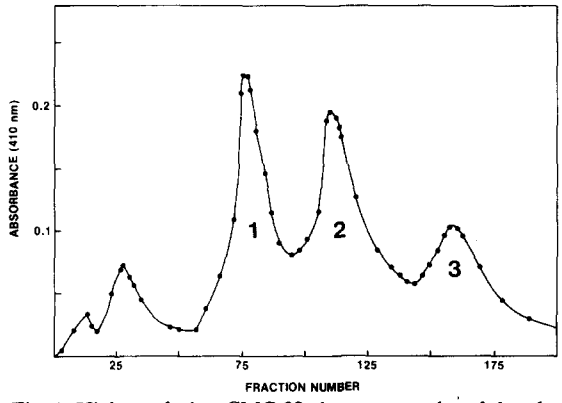

Fig. 1. High-resolution CMC-32 chromatography of the photooxidation reaction products of Cyt *c* pH 7.5 50 mM phosphate; 1.5 cm×32 cm column, 7.5 ml/fraction, 0.5 ml/min.

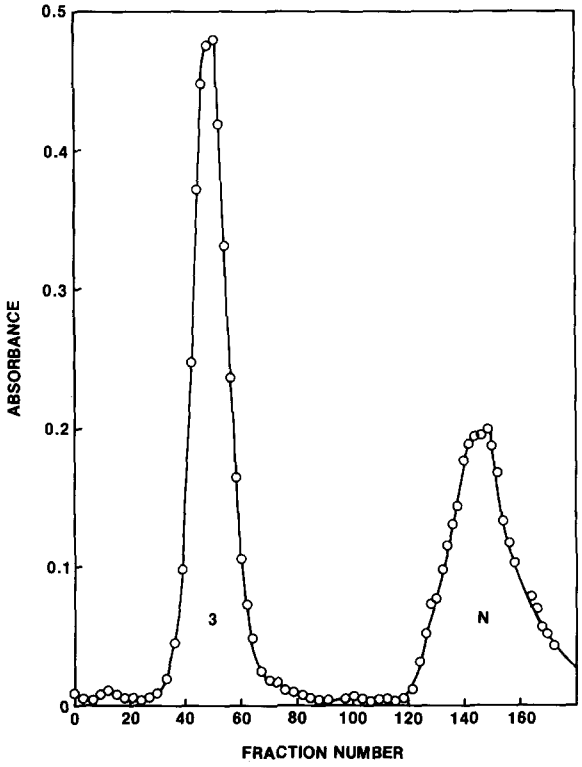

Fig. 2. CMC-32 co-chromatography of native Cyt *c* with protein fraction 3 (see Fig. 1). pH 7.5, 50 mM phosphate; 1.5 cm × 32 cm column, 7 ml/fraction, 0.3 ml/min.

chrome *c*. The results shown in Fig. 2 indicate that native protein (verified as native by an intact 695 nm band) is eluted far after all other fractions and would have been observed if any of it remained after modification. When the same sample of photooxidized cytochrome *c* was subjected to Amberlite IRC-50 chromatography as described by Ivanetich et al. [13] essentially no chromatographic resolution was obtained, i.e., a single peak with a poorly resolved shoulder on each side of it was observed, thus giving under these conditions the erroneous impression that the modified cytochrome *c* is a homogeneous product.

Using the spectroelectrochemical methods of Heineman [15], the formal reduction potentials, $E^{0\prime}_{7.0}$, were obtained through repeated measurements for the three main proteins obtained from CMC chromatography. The experimental titration curves for all three fractions and for native Cyt *c* are shown in Fig. 3, and the $E^{0\prime}$ values of each fraction are summarized in Table I. With the

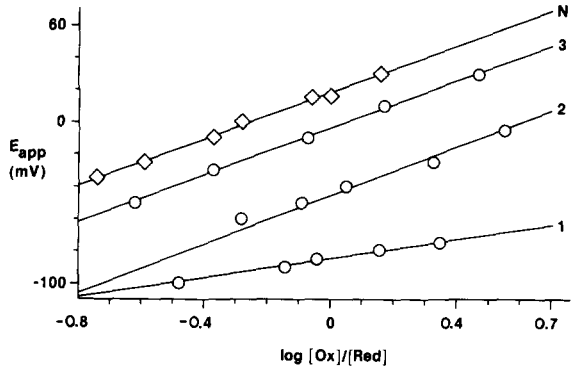

Fig. 3. Spectroelectrochemically determined titration curves, $E_{applied}$ vs. log (Cyt $c_{oxidized}$)/(Cyt $c_{reduced}$), of native Cyt *c* (*N*) and the main fractions 1, 2 and 3 from analytical chromatography shown in Fig. 1. The $E^{0\prime}$'s obtained from the titration are 262 mV for native Cyt *c*, and 161 mV, 202 mV and 240 mV for fractions 1, 2, and 3, respectively.

exception of protein fraction 1 (the most highly modified Cyt *c* derivative), where $n = 2$, the *n* values (number of electrons transferred to and from the cytochrome *c* per mole) obtained from the titration experiments were equal to unity. The $E^{0\prime}$ determined for native Cyt *c* was found to be 262 mV, the accepted literature value. In Table I are also shown the relative amounts of each protein fraction; from this information the weighted average $E^{0\prime}$ for the mixture was calculated to be $E^{0\prime}_{\text{weighted average}} = 191$ mV vs. N.H.E. Weighted redox potentials of mixtures of related redox molecules have been used and described by Taylor and Schilt [18].

Spectroscopically, all three protein fractions in the oxidized state were missing the absorption peak at 695 nm, thus indicating a change in the

TABLE I

RELATIVE INTEGRATION OF CHROMATOGRAPHIC PEAKS (SEE FIG. 1) AND ASSOCIATED REDUCTION POTENTIALS FOR CHROMATOGRAPHICALLY SEPARATED METHIONINE SULFOXIDE PRODUCTS

$E^{0\prime}_{\text{weighted average}} = 191$ mV.

Protein fraction	$E^{0\prime}$ (mV)	Relative integration
1	161	0.37
2	202	0.40
3	240	0.23

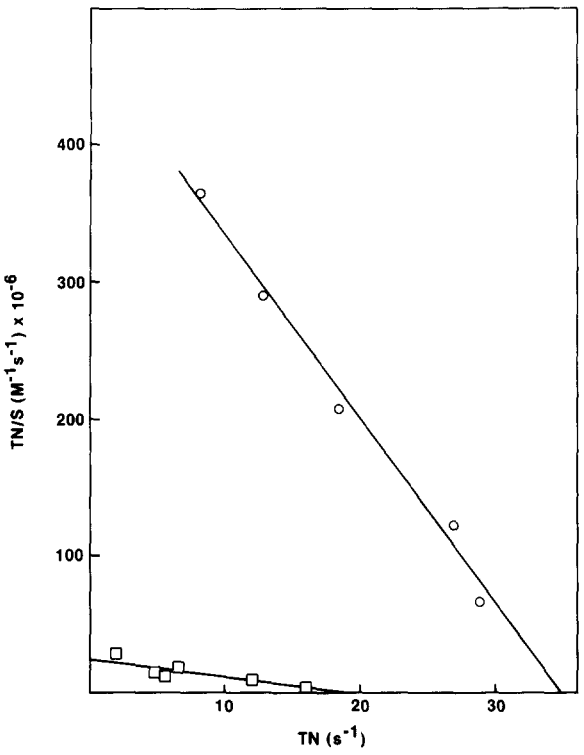

Fig. 4. Succinate Cyt *c* reductase activity of native and methionine-80 sulfoxide Cyt *c*, determined polarographically using bovine heart mitochondrial particles (Keilin-Hartree particles). pH 7.9, 0.1 nmol cytochrome aa_3, 14 mM succinate, total volume 1.8 ml. Open circles (○) for native horse heart Cyt *c* and open squares (□) for methionine-80-sulfoxide Cyt *c*.

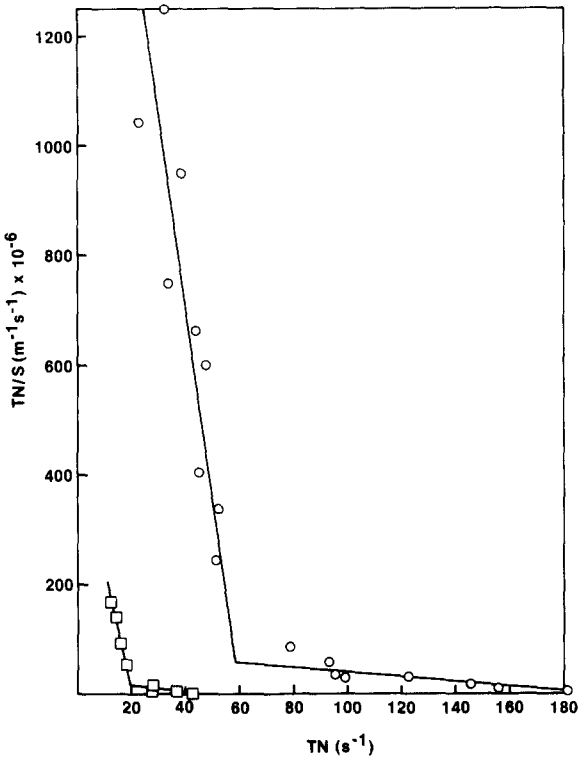

Fig. 5. Ascorbate-TMPD-cytochrome *c* oxidase activity determined polarographically. Experimental conditions: 25 mM Tris cacodylate (pH 7.9)/0.08 nmol aa_3, and total volume 1.8 ml. Open circles (○) for native horse heart Cyt *c* and open squares (□) for methionine-80-sulfoxide Cyt *c*.

Met-80 S-Fe coordination. In light of previous experience with modified cytochromes *c* [19], the co-chromatography experiments described above, and the $E^{0\prime}$ of each fractions, fraction 3 appeared to be the most similar (least modified) to native cytochrome *c*. Thus, amino acid analysis of fraction 3 was performed [10]. Only one of the two possible methionines was modified, and then as the sulfoxide.

The reaction products of the photooxidation of Cyt *c* were subjected to isoelectrofocusing after being partially purified with Amberlite IRC-50 as described by Ivanetich et al. [13]. This crude product contained all the protein fractions as obtained in the analytical chromatography shown in Fig. 1. Isoelectrofocusing resulted in single main peak with two slight shoulders on its right, thus indicating that the protein fractions have similar pI's.

In Fig. 4 is shown the comparison of the native

TABLE II

SUMMARY OF SUCCINATE CYT *c* REDUCTASE AND ASCORBATE-TMPD-OXIDASE ACTIVITIES FOR NATIVE AND Met-80-SULFOXIDE CYTOCHROME *c*

See Experimental Methods for conditions used.

		K_m (M)	TN_{max} (s^{-1})
Reductase activity	native	$8 \cdot 10^{-8}$	35
	Met-80	$7 \cdot 10^{-7}$	19
Oxidase activity			
Low-concentration region			
Tris/pH 7.9	native	$3 \cdot 10^{-8}$	60
	Met-80	$4.4 \cdot 10^{-8}$	20
Phosphate/pH 6.5	native	$1.0 \cdot 10^{-7}$	58
	Met-80	$2.0 \cdot 10^{-7}$	20
High-concentration region			
Tris/pH 7.9	native	$2.3 \cdot 10^{-6}$	190
	Met-80	$1.8 \cdot 10^{-6}$	47
Phosphate/pH 6.5	native	$5 \cdot 10^{-6}$	500
	Met-80	$1.6 \cdot 10^{-5}$	200

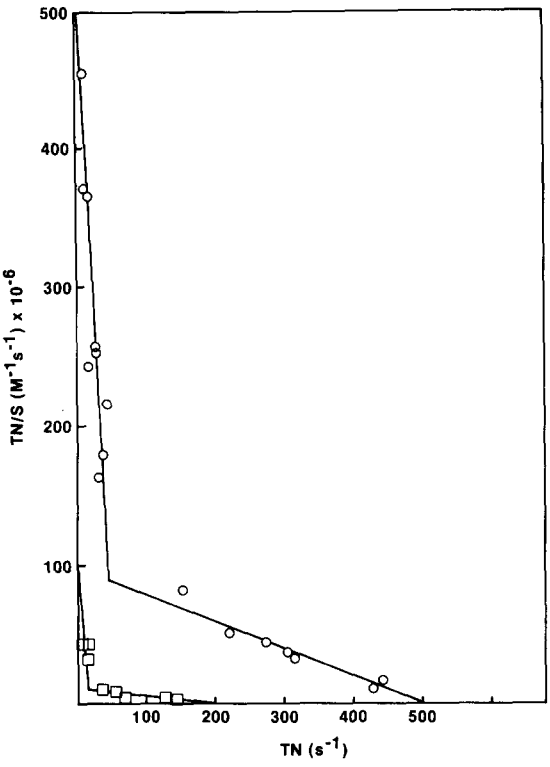

Fig. 6. Ascorbate-TMPD-cytochrome *c* oxidase activity determined polarographically. Experimental conditions: 50 mM phosphate buffer (pH 6.5)/0.02 nmol Cyt aa_3, and total volume 1.8 ml. Open circles (○) for native horse heart Cyt *c* and open squares (□) for methionine-80-sulfoxide Cyt *c*.

and methionine-80-sulfoxide succinate-cytochrome *c* reductase activity measured with bovine heart mitochondrial particles. Although rates of oxygen uptake are monitored, the rate-limiting reaction is the reduction of the Cyt *c* by Cyt c_1, and cytochrome oxidase acts as the coupled assay system [17]. In Fig. 5 and 6 are shown the comparison of the ascorbate-TMPD-cytochrome *c* oxidase activity of the same mitochondrial particles examined under two sets of conditions, respectively, 25 mM Tris cacodylate (pH 7.9 mM) and 50 mM phosphate (pH 6.5) (see Experimental Methods). All oxidase and reductase activities were determined polarographically and provided the apparent Michaelis constants, K_m, and maximum turnover numbers, TN_{max}. These results are summarized in Table II.

Discussion

The analytical high resolution CMC-32 chromatography, shown in Fig. 1, demonstrated unequivocally that when using the procedures described by Ivanetich et al. [13], a mixture of modified cytochromes *c* is obtained. Also, since all the native protein reacts and since all fractions were missing the 695 nm absorption band, it is probable that all three fractions do indeed have the Met-80 as the sulfoxide. That the preparation provides a mixture was missed in earlier work [13], apparently since higher concentrations of buffer were used which in turn eliminated any chromatographic resolution of the components. Co-chromatography of protein fraction 3 with native Cyt *c* (see Fig. 2) indicated that the charge distribution Cyt *c* molecular surface (responsible for the observed chromatographic behavior) of fraction 3 was the most similar to native protein and thus the least modified internally and externally [19]. It is for this reason that the amino acid analysis was done on fraction 3 with the result that only one of the two methionines was modified [10]. Since only one methionine was modified, and since the 695 nm band was also missing in the visible spectrum of the oxidized protein, it was concluded that fraction 3 is the methionine-80-sulfoxide Cyt *c*. In regard to protein fractions 1 and 2, their chromatographic behavior and low redox potentials indicate extensive internal and/or external modification.

The potentials of fractions 1–3 were determined to be: 161 mV, 202 mV and 240 mV, respectively (see Fig. 4). Clearly, since there is no evidence that a simple modification of the external Met 65 should result in extensive protein structural change and subsequent $E^{0\prime}$ change, then fractions 1 and 2 again probably have residues that are modified above and beyond Met-65 and Met-80. The redox potentials of fractions 1 and 2 are typical of cytochromes that have been extensively modified and were not explored further.

When the $E^{0\prime}$'s for all three fraction shown in Fig. 1 were weighted according to their integration (see Table I), an $E^{0\prime}_{\text{weighted average}} = 191$ mV was obtained. This weighted $E^{0\prime}$ is very similar to the redox potential, $E^{0\prime} = 184$ mV, reported by Ivanetich et al. [13] for the 'methionine-80 sulfo-

xide Cyt. *c*'. Subsequently, Myer et al. [20] have used chloramine-T to modify Cyt *c* and presumably obtained the Met-80-sulfoxide-Met-65-sulfoxide Cyt *c* with a redox potential of $E^{0\prime} = 170$ mV. Again this low potential is very close to our $E^{0\prime}_{\text{weighted average}}$ but inconsistent with the $E^{0\prime} = 240$ mV of the chromatographically homogeneous, amino acid analyzed Met-80-sulfoxide Cyt *c* obtained in this work. An $E^{0\prime}$ of 170 mV is indicative of a mixture of sulfoxide cytochromes *c*. Also in their work [20] Amberlite IRC-50 was again used [21] as in Ivanetich's work and with eluting rather than chromatographing concentrations of salt. It is probable that the preparation by Myer et al. [20] is also a mixture of Met-80 sulfoxide products. This might explain that while the results discussed below for reductase activity are very similar to those obtained by Myer et al. [20] on the Chloramine-T prepared methionine-80-sulfoxide Cyt *c*, the oxidase activity with this methionine-80 sulfoxide [20] and with native protein gave about the same apparent K_m and turnover number (V_{max}).

The isoelectrofocusing of the reaction products as purified by IRC-50 resulted in a main peak with poorly resolved shoulders, but all with about the same p*I*. This is not unexpected, since the formation of the sulfoxide results in an isoelectric product, i.e., one that results in no net change of methionine-80 charge. The observed p*I* for the modified protein band was 10.08. This means that isoelectrofocusing of the methionine-sulfoxide product is not an especially effective test of purity. It is probable that in studies by Brittain and Greenwood [22] were done on a mixture of cytochromes *c* even though they indicated that isoelectrofocusing gave one band. Although they used carboxymethyl cellulose for chromatography, the crude product was apparently eluted rather than chromatographed with 0.2 M Tris (or 0.1 M cacodylate/HCl).

Biological activity

Biophysical characterization of the Met-80-sulfoxide Cyt *c* prepared in this work was reported earlier and was accomplished by examining cyanide reactivity of the modified Cyt *c* as compared to the native protein [10]. The ability of the Met-80-sulfoxide Cyt *c* to transfer electrons was evaluated via the reactivity of the native and modified Cyt *c* with the succinate-Cyt *c* reductase (Fig. 4) and ascorbate-TMPD-Cyt *c*-oxidase activities shown in Figs. 5 and 6. The quantitative aspects of these results are summarized in Table II. For reductase activity, both the apparent K_m and the maximal turnover number are dramatically affected with native vs. modified protein. The K_m for native and modified cytochrome *c* are $8 \cdot 10^{-8}$ M and $7 \cdot 10^{-7}$ M, respectively. This means that the Met-80-sulfoxide Cyt *c* binds to the reductase about nine-times less strongly than native Cyt *c*. Additionally, reductase TN_{max} with the modified protein (19 s^{-1}) is only about 54% of the reductase TN_{max} with native Cyt *c* (35 s^{-1}). These results are reasonable when it is noted that it is oxidized Cyt *c* that binds to the reductase. This redox form of the cytochrome has the more 'open' conformation and is likely to be more sensitive to modification of residues involved in determining the structure of the heme crevice. The loss of the 695 nm band noted above indicates that the heme crevice of Cyt *c* has probably opened further after conversion of Met-80 to the sulfoxide, with resultant changes in the conformation of the areas involved in the binding to reductase. The lower redox potential of the Met-80 sulfoxide Cyt *c* might also contribute to a slower rate of electron transfer and thus lower turnover number.

An examination of the results for ascorbate-TMPD-cytochrome *c* oxidase activity shown in Figs. 5 and 6 and Table II provide a strong contrast to the reductase activity. Under two different sets of experimental conditions, the modified cytochrome *c* shows the same biphasic behavior that is typical of native cytochrome *c*. In examining the low concentration phase of the activity in Tris cacodylate buffer (see Fig. 5), the K_m's are nearly the same (i.e., modified, $K_m = 4.4 \cdot 10^{-8}$ M vs. native, $K_m = 3 \cdot 10^{-8}$), while the maximal turnover numbers are quite different. Oxidase activity with the modified protein ($TN_{max} = 20$ s^{-1}) was about 3-times slower than with the native protein ($TN_{max} = 60$ s^{-1}). The high concentration phase of activity also gave similar K_m's (modified, $K_m = 1.8 \cdot 10^{-6}$ M vs. native, $K_m = 2.3 \cdot 10^{-6}$ M). In contrast oxidase activity with modified protein ($TN_{max} = 47$ s^{-1}) was about 4-times

slower than with the modified protein (TN_{max} = 190 s^{-1}).

The results obtained with Tris buffer were confirmed by additional oxidase experiments in phosphate buffer (pH 6.5) shown in Fig. 6 and in Table II. In the low concentration phase the K_m's for modified and native protein were again similar ($2.0 \cdot 10^{-7}$ M and $1.0 \cdot 10^{-7}$ M, respectively) and oxidase maximal turnover numbers with modified protein being about 3-times slower than with native (20 s^{-1} vs. 58 s^{-1}). In the high concentration phase the K_m's with modified and native protein were also similar ($1.6 \cdot 10^{-6}$ M vs. $5.0 \cdot 10^{-6}$ M), with the maximal turnover number with modified protein being 2.5-times slower than with native protein (200 s^{-1} vs. 500 s^{-1}). The experiments done in phosphate are especially important when it is noted that the maximal velocities achieved in the polarographic assay at pH 6.5 in 50 mM phosphate are very similar to those obtained when the enzyme is assayed spectrophotometrically under the same conditions [23], indicating that neither TMPD nor ascorbate is rate limiting.

The Cyt *c* oxidase results, especially those shown in Fig. 5, are particularly interesting, since it is primarily the rate of electron transfer that is altered and not the binding of reduced modified Cyt *c* to the oxidase. In spite of the modification of Met-80, the reduced Met-80-cytochrome *c* is apparently able to achieve a conformation close to normal and, unlike the oxidized form, form a proper complex with oxidase. What is then observed is a significantly slower electron-transfer rate for the Met-80-sulfoxide Cyt *c*. These results are consistent with the model work of Mashiko [5] and indicate that the parity of acid/base and electrostatic interactions of the Met 80-Fe coordination bond have been disrupted in going from native Cyt *c* to Met-80-sulfoxide Cyt *c*. This in turn results in the observed slower electron-transfer rates. Once the parity of these interactions is disrupted, significant reorganizations of the metal coordination sphere may be required and occur when going from one redox state to the other [8].

Acknowledgements

This work was supported by the American Chemical Society-Petroleum Research Fund Grant (ACS-PRF 8883-G4) and a University of Wisconsin-Milwaukeee Graduate School Research Award to B.A.F. and a grant from the National Institutes of Health (GM 26916) to S.F.M.

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