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Methionine Sulfoxide Cytochrome *c*†

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ABSTRACT: Cytochrome *c* has been chemically modified by methylene blue mediated photooxidation. It is established that the methionine residues of the protein have been specifically converted to methionine sulfoxide residues. No oxidation of any other amino acid residues or the cysteine thioether bridges of the molecule occurs during the photooxidation reaction. The absorbance spectrum of methionine sulfoxide ferricytochrome *c* at neutrality is similar to that of the unmodified protein except for an increase in the extinction coefficient of the Soret absorbance band and for the complete loss of the ligand sensitive 695 nm absorbance band in the spectrum of the derivative. The protein remains in the low spin configuration which implies the retention of two strong field ligands. Spin state sensitive spectral titrations and model studies of heme peptides indicate that the sixth ligand is definitely not provided by a lysine residue but may be methionine-80 sulfoxide coordinated via its sulfur atom. Circular dichroism spectra indicate that the heme crevice of methionine sulfoxide ferri- and ferrocytochrome *c* is weakened relative to native cytochrome *c*. The redox potential of methionine sulfoxide cytochrome *c* is 184 mV which is markedly diminished from the 260 mV redox potential of native cytochrome *c*. The modified protein is equivalent to native cytochrome *c* as a substrate for cytochrome oxidase and is not autoxidizable at neutral pH but is virtually inactive with succinate-cytochrome *c* reductase. These results indicate that the major role of the methionine-80 in cytochrome *c* is to preserve a closed hydrophobic heme crevice which is essential for the maintainance of the necessary redox potential.

Cytochrome *c* is a heme protein which transfers single electrons from cytochrome c_1 to cytochrome oxidase in the mitochondrial respiratory chain. The protein is composed of a polypeptide of approximately 100 amino acid residues and an iron protoporphyrin IX (heme) prosthetic group (Margoliash and Schejter, 1966; Dayhoff and Eck, 1973) which is capable of undergoing alternate oxidation and reduction of its central iron ion. The heme moiety of cytochrome *c* is covalently bonded to the protein via two cysteine thioether bridges and via coordination of histidine residue 18 and methionine residue 80 of the protein as the fifth and sixth (axial) ligands to the heme iron (for a review see Lemberg and Barrett, 1973).

The mechanism of action of cytochrome *c* in the mitochondrial electron transfer chain remains to be elucidated. Electron transfer to the heme iron of cytochrome *c* has been proposed to occur via peripheral attack on the solvent exposed edge of the heme (Dickerson et al., 1971; Sutin and Yandell, 1972) or via transfer through a portion of the pro-

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tein to an axial ligand of the heme iron (Winfield, 1965; Dickerson et al., 1972). The sixth (axial) ligand, methionine-80, has proved to be of particular importance in attempts to establish whether electron transfer to and from cytochrome *c* occurs via the protein donated ligands to the heme iron. Alterations in the strength of liganding of this residue to the heme iron with changes in the oxidation state of the heme iron of cytochrome *c* are well documented (Harbury et al., 1965; Lemberg and Barrett, 1973). Disruption of the methionine-80–heme iron bond invariably results in alterations in the electron transfer properties of cytochrome *c* (Stellwagen, 1968; Horecker and Kornberg, 1946; Schejter and George, 1964; Margoliash et al., 1973).

The recent report that methionine-80 sulfoxide cytochrome *c* functions almost as well as cytochrome *c* in transferring electrons in the mitochondrion (Folin et al., 1972) provided the first example of a chemical modification of methionine-80 that did not produce major alterations in the electron transfer function of the molecule. Methionine-80 sulfoxide cytochrome *c* is therefore eminently suitable for investigations into the role of methionine-80 in electron transfer to and from the heme iron atom of cytochrome *c*. To further investigate the role of methionine-80 in electron transfer by cytochrome *c*, we attempted to repeat the synthesis of methionine-80 sulfoxide cytochrome *c* reported by Jori et al. (1970). The inability to repeat this synthesis in our and in several other laboratories (Y. P. Myer, personal communication; E. Stellwagen, personal communication; E. Margoliash, personal communication; A. Schejter, personal communication; H. A. Harbury, personal communication) prompted us to devise an alternate synthesis for this compound. We report here on the preparation, characterization, and electron transfer properties of methionine sulfoxide cytochrome *c*.

Experimental Procedure

Materials. Horse heart cytochrome *c* was obtained from Miles Laboratories (Grade I), and in a few experiments the Sigma Type III product was utilized after purification on an Amberlite CG-50 column (Margoliash and Walasek, 1967). Cytochrome *c* was oxidized at the two thioether bridges as described by Lederer and Tarin (1971). A heme undecapeptide was prepared from horse heart cytochrome *c* as described by Harbury and Loach (1960). Cytochrome oxidase was isolated from fresh beef heart mitochondria by the method of Yonetani (1967). Succinate-cytochrome *c* reductase was a gift of Drs. C. A. Yu and T. E. King, prepared by the method of Yu et al. (1974). L-Methionine and L-methionine DL-sulfoxide were products of Sigma Chemicals. Potassium ferrocyanide was Analar grade from Riedel-De Häen. Methylene blue was obtained from M and B Chemicals. Silica gel G thin-layer plates (0.25 mm × 5 cm × 20 cm) without fluorescent indicator were purchased from Merck. *N*-Acetylmethionine sulfoxide was prepared by the reaction of 3.82 g (0.02 mol) of *N*-acetylmethionine with 4.28 g (0.02 mol) of sodium metaperiodate (Fluka AG) in 23% (v/v) aqueous methanol at 0 °C. The reaction was stirred for 5 h at 0–5 °C and subsequently for 14 h at room temperature. The purified material chromatographed on silica gel G thin-layer plates in isopropyl alcohol–ethyl acetate–17% ammonia (2:2:1, v/v) with an R_f of 0.18 (compared to an R_f of 0.30 for *N*-acetylmethionine). The product did not react with ninhydrin and after basic hydrolysis (Noltmann et al., 1962) reverted in over 85% yield to methionine sulfoxide as estimated by automatic amino acid analysis.

Methods. Numerous attempts were made to photooxidize the methionine residue at position 80 of horse heart ferricytochrome *c* without added photosensitizer. In some experiments, the procedure of Jori et al. (1970) was followed exactly. In other experiments, the following modifications were introduced. (1) Irradiation time: 1–60 min. (2) Buffer conditions: pH 2.2, 5.1–8.2; distilled water (three sources), deionized water, various buffer salts. (3) Horse heart cytochrome *c*: Miles Laboratories (Type I, 95% pure, 0.425% Fe) or Sigma Chemicals (Type III) used as received or oxidized with potassium ferricyanide and purified on Amberlite CG-50 or Sephadex G-75 (Margoliash and Lustgarten, 1962). Purified samples were used fresh in the solution in which they were chromatographed or dialyzed and lyophilized. (4) Lamps: four 300-W Tungsten lamps (Phillips G74) operated at 220 V (or at 380 V in one experiment to simulate the energy spectrum of 300 W–120 V lamps operated at 120 V) and/or one 1000-W Mercury lamp (Phillips HPL-N1000W,57222G/74) with or without reflectors. (5) Distance of lamps from sample: 5–40 cm. (6) Reaction vessel: quartz cuvette, glass test tubes, a water jacketed test tube (1.2 × 23 cm), flat petri dish (irradiated from above), thermostated or not. (7) Reaction temperature: 0–45 °C. (8) Oxygen source: two tanks of pure O_2, one tank 90:10 O_2–CO_2. Attempts to photooxidize ferricytochrome *c* in 0.1 M phosphate buffer and ferrocytochrome *c* in 0.05 M phosphate buffer, pH 8.2 and pH 11.1, without added photosensitizer were performed following the method of Jori et al. (1970, 1971).

The photosensitizer methylene blue was investigated as a mediator of the oxidation of methionine in the following experiment.

A solution of 1 mM L-methionine in 0.1 mM methylene blue in 99% acetic acid was irradiated at 37 °C for 30 min with four 300-W Tungsten lamps fitted with reflectors. The lamps were located 20 cm from the thermostated reaction vessel a glass test tube (1.2 × 23 cm) enclosed in a glass water jacket (3.2 × 28 cm). Oxygen was slowly fluxed through the solution for the duration of the reaction. To monitor the photooxidation of methionine, methionine and methionine sulfoxide were separated by chromatography on silica gel G thin-layer plates in ethyl acetate–isopropyl alcohol–17% ammonia (2:2:1, v/v) and estimated visually after spraying the developed chromatograms with 0.1% ninhydrin in 50% (v/v) aqueous acetone. The reaction of L-methionine DL-sulfoxide (2.6 mM) with sodium dithionite (26 mM) was conducted at room temperature for 12 h, and the reaction mixture was chromatographed as described above.

Methylene blue was then used in the photooxidation of cytochrome *c* as follows. A solution of 33 mg of ferricytochrome *c* (1.1×10^{-4} M) and 0.78 mg of methylene blue (8.7×10^{-4} M) in 24 ml of 84% (v/v) acetic acid was irradiated for reaction times of 2 min to 4 h at 0–4 °C. The experimental details are as described above for the photooxidation of methionine. The reaction solution was lyophilized and subsequently chromatographed on a 2.5 cm × 35 cm column of Sephadex G-75 equilibrated with 0.05 M ammonium acetate (pH 7.0). The major protein band was eluted and the fractions corresponding to this band were analyzed immediately or lyophilized twice and stored at −40 °C. Any front running polymeric material was discarded.

Cytochrome *c*, treated as above but not irradiated, was retained in darkness for 16 h and was used as a reaction blank. Unless otherwise indicated, photooxidized cyto-

chrome *c* (or methionine sulfoxide cytochrome *c*) refers to samples of ferricytochrome *c* photooxidized in the presence of methylene blue for between 60 and 90 min and subsequently purified. Our criterion for an acceptable sample was that less than 5% of the 695-nm band was retained. Photooxidized ferrocytochrome *c* (or methionine sulfoxide ferrocytochrome *c*) refers to methionine sulfoxide cytochrome *c* in which the iron was reduced with sodium dithionite after photooxidation of the protein. The reductant was removed from this derivative by chromatography on a Sephadex G-25M column (0.9 × 18 cm) in an appropriate buffer.

Amino acid analyses were performed on a Beckman Model 120C amino acid analyzer. Samples were hydrolyzed at 110 °C for 24 h in 2 M barium hydroxide (Noltmann et al., 1962) to determine methionine, methionine sulfoxide, and tryptophan or hydrolyzed in redistilled constant boiling hydrochloric acid to determine all other amino acids.

To locate and quantitate the methionine sulfoxide residues of photooxidized cytochrome *c*, 30 mg of protein was treated with a 250-fold excess of cyanogen bromide (relative to protein) for 16–20 h (Corradin and Harbury, 1970) and subsequently treated with dinitrofluorobenzene (Narita, 1970). The resulting Dnp-peptides[1] were hydrolyzed in constant boiling hydrochloric acid for 12 h at 110 °C. The Dnp-amino acids were purified (Narita, 1970) and separated by thin-layer chromatography on silica gel G plates (25 cm × 5 cm × 0.25 mm) in a solvent system of benzene–pyridine–glacial acetic acid (80:20:2, v/v). Dnp-Glu and Dnp-Ile exhibited R_f values of 0.21 and 0.85, respectively. The purified Dnp-amino acids were quantitated as described by Narita (1970). The amount of each methionine residue modified was calculated from the ratio of methionine-80 to methionine-65 (from the cyanogen bromide data) and the total number of methionine residues modified (determined by amino acid analysis).

The degree of photooxidation of the thioether bridges of the protein was estimated by the following procedure (F. Lederer, personal communication): 3 mg of ferricytochrome *c* or derivative (0.24 μmol) was incubated in 0.2 ml of 8 M urea for 24 h under nitrogen. Then 0.45 ml of formic acid and 10.6 mg of cyanogen bromide (500-fold excess to protein) were added and the reaction was allowed to proceed for 6–24 h at room temperature. The reaction solution was then chromatographed and the amount of cleavage of the cysteine thioether bridges estimated as described by Lederer and Tarin (1971).

The absorbance of the 695-nm band of ferricytochrome *c* and derivatives was determined by subtracting the residual absorbance of the broad absorbance band at 530 nm from the observed absorbance at 695 nm (Kaminsky et al., 1973). The calculated absorbance of a fully developed 695-nm absorbance band (corrected for background absorbance) was determined using $E_{695\text{ nm band}}$(1 cm) 225. The concentrations of cytochrome *c* and derivatives were calculated from the absorbance at 530 nm where $E_{530\text{ nm}}$(1 cm) 1.12×10^4 (Margoliash and Frohwirt, 1959) or from the pyridine hemochrome $E_{550\text{ nm}}$(1 cm) 2.91×10^4 (Norton, 1958). Circular dichroism spectra were recorded using a Jasco J-40A automatic recording spectropolarimeter operated at constant resolution. Solutions of ferricytochrome *c* and photooxidized ferricytochrome *c* varied from 7×10^{-5} to 1.4×10^{-4} M in 0.05 M potassium phosphate buffer (pH 7.5 and pH 5.0). Solutions of ferrocytochrome *c* and methionine sulfoxide ferrocytochrome *c* were 12 μM and contained sodium dithionite to maintain them in the fully reduced state. All solutions were filtered through Millipore filters (0.45 μ) prior to spectral studies. Spectra were recorded at 20 °C in cuvettes of 1- and 10-mm path lengths.

The reaction of cytochrome oxidase with unmodified and methionine sulfoxide ferrocytochromes *c* was studied in 0.1 M phosphate buffer (pH 5.8 and pH 7.5) at 25 °C (Minnaert, 1961). The autoxidation and cytochrome oxidase catalyzed oxidation reactions of ferrocytochrome *c* and derivatives were monitored spectrally at 550 nm.

The reducibility of cytochrome *c* and methionine sulfoxide cytochrome *c* by solubilized succinate-cytochrome *c* reductase was measured according to the method of Takemori and King (1964). The assays were performed in 0.1 M phosphate buffer–0.3 mM EDTA (pH 7.4) with 20 mM sodium succinate as the electron donor.

Unmodified and methionine sulfoxide cytochromes *c* (0.5–1.0 μmol) were oxidized with potassium ferricyanide and chromatographed on an Amberlite CG-50 column (1.2 × 37 cm) with sodium phosphate buffer (Na concentration 0.34 M) (pH 8.7). Each sample eluted as a single band, with cytochrome *c* and the oxidized derivative exhibiting elution volumes of 95 and 115 ml, respectively.

The redox potentials of methionine sulfoxide cytochrome *c* and native cytochrome *c* were determined by the method of Brandt et al. (1966) utilizing the spectrally determined equilibrium constants for the reaction of ferricytochrome *c* or methionine sulfoxide ferricytochrome *c* with potassium ferrocyanide. This equilibrium reaction was studied at pH 7.0 under anaerobic conditions. Ion association was corrected for in calculating the results (Kolthoff and Tomsicek, 1935; Eaton et al., 1967).

The interaction of the heme undecapeptide ($2\text{–}10 \times 10^{-5}$ M) and various sulfur compounds (1.0–2.5 M) was monitored spectrally in 4-cm path-length cuvettes at room temperature. All spectral studies were performed on a Unicam SP1800 spectrophotometer with SP1805 program controller.

Results

All attempts to photooxidize methionine-80 of ferricytochrome *c* in the absence of added photosensitizer as per Jori et al. (1970) failed. None of the modifications which were introduced into the reported procedure (see Methods) resulted in any decrease in the methionine content of the protein or in the loss of the 695-nm absorbance band of ferricytochrome *c*. Additional attempts to photooxidize cytochrome *c* without added photosensitizer, under conditions reported to modify methionine-80 and histidine-18 as well as other photosensitive amino acids (Jori et al., 1970, 1971), also did not result in any loss of the 695-nm absorbance band of the protein.

Methylene blue mediated photooxidation of L-methionine was complete within 30 min, at which time methionine was entirely converted to methionine sulfoxide. Photooxidation of ferricytochrome *c* in the presence of methylene blue for reaction times of 2 min to 2 h resulted in modification of only the methionine residues of the protein (Tables I and II).

A plot of the percent of the 695-nm absorbance band retained by solutions of photooxidized cytochrome *c* vs. the reaction time of methylene blue mediated photooxidation (Figure 1) demonstrates that the 695-nm absorbance band

[1] Abbreviation used is: Dnp, dinitrophenyl.

Table I: Amino Acid Analyses of Barium Hydroxide Hydrolysates of Native and Photooxidized Cytochromes *c*.

Pretreatment of Cytochrome *c*			Amino Acid Analysis				
Photoox.	Photosensitizer	Rxn Time (min)	Trp[a]	Met	Tyr	Phe	MSO[b]
No			0.9	2.0	3.5	3.6	0.0
Yes	None	1-60	0.9	2.0	3.5	3.8	0.0
No	MB[b]		0.9	2.1	3.5	3.6	0.0
Yes	MB	2	0.9	1.6	3.7	3.4	0.4[c]
Yes	MB	60	0.8	0.6	4.0	3.6	1.4
Yes[d]	MB	60	0.7	0.8	3.5	3.8	1.2[c]
Yes[e]	MB	60	0.6	0.8	3.7	4.0	1.2
Yes	MB	120	0.8	0.4	3.5	3.7	1.6[c]
Yes	MB	240	0.7	0.2	4.0	3.8	1.8[c]

[a] Based on lysine = 19.0. [b] Abbreviations used are: MSO, methionine sulfoxide; MB, methylene blue. [c] Calculated from difference between 2.0 and the determined no. of residues of Met. [d] Photooxidized cytochrome *c* was reduced with sodium dithionite prior to analysis. [e] Photooxidized cytochrome *c* was reduced with sodium dithionite and subsequently oxidized by cytochrome oxidase prior to analysis.

Table II: Amino Acid Analyses of Hydrochloric Acid Hydrolysates of Native and Photooxidized Cytochromes *c*.

	Cytochrome *c* Sample			
Amino Acid	Unmodified	Blank[a]	Photoox. 1 h[b]	*Photoox.* 4 h[b]
Asp	8.2 (8)	8.3	8.2	8.3
Thr	9.6 (10)	9.8	10.0	10.1
Ser	(0)			Shoulder
Pro	4.2 (4)	4.1	4.1	4.3
Glu	12.3 (12)	12.6	12.5	12.6
Gly	12.5 (12)	13.5	12.7	12.6
Ala	6.3 (6)	6.5	7.0	6.8
Cys	1.2 (2)	1.1	1.1	1.2
Val	3.0 (3)	3.0	3.0	3.1
Met[c]	1.9 (2)	1.8	2.0	2.0
Ile	5.9 (6)	5.8	5.7	5.8
Leu	6.1 (6)	6.3	6.2	6.4
Tyr	3.7 (4)	3.8	3.7	3.6
Phe	3.9 (4)	4.0	3.9	3.9
Lys	19.0 (19)	18.6	18.5	19.0
His	3.1 (3)	2.9	3.1	3.2
Arg	2.1 (2)	2.1	2.0	2.0

[a] See Methods. [b] In the presence of methylene blue. [c] Methionine sulfoxide reverts to methionine during acid hydrolysis (Ray and Koshland, 1962).

decreases with increasing reaction time and is essentially completely lost after 1 h of reaction. The results of the cyanogen bromide cleavage and subsequent dinitrofluorobenzene treatment of photooxidized cytochrome *c* indicate that methionine-80 is photooxidized to methionine sulfoxide more rapidly than is methionine-65 (Table III). After 1 h of reaction, modification of methionine-80 is over 80% complete while approximately 60% of the methionine-65 is modified.

Additional cyanogen bromide cleavage experiments were performed to determine if the thioether bridges to the heme moiety had undergone any oxidation during the course of the photooxidation reaction. The results of these experiments are presented in Table IV. No decrease in the ratio of heme to protein is apparent in samples photooxidized for 1 h.

Table III: Determination of Methionine Sulfoxide Residues in Photooxidized Cytochrome *c*.

Cytochrome *c* Sample[a]	Dnp-Ile/Dnp-Glu[b,c]	No. Residues Photooxidized	
		Met-65	Met-80
Blank[d]	1.0:0.9	0.0	0.0
Photooxidized[e]	0.5:1.0	0.6	0.8

[a] The samples were treated with cyanogen bromide and subsequently with dinitrofluorobenzene prior to analysis (see Methods). [b] Dnp, dinitrophenyl. [c] N-terminal isoleucine and glutamic acid are produced when cyanogen bromide cleaves after methionine-80 and methionine-65, respectively. Cyanogen bromide does not cleave after methionine sulfoxide in peptides. [d] See Methods. [e] Photooxidized for 1 h.

Table IV: Determination of the Extent of Oxidation of the Cysteine Thioether Bridges of Cytochrome *c* and Derivatives.

Cytochrome *c* Derivative	$A_{398\ nm}/A_{280\ nm}$	% Cys Thioether Sulfoxide
Unmodified[a]	5.7 ± 0.3	0
Unmodified[a,b]	5.4	0
Unmodified[b]	6.1 ± 1.2	0
Unmodified	6.1 ± 1.1	0
Photooxidized 2 min	5.5 ± 1.1	0
Photooxidized 1 h[a]	6.0	0
Photooxidized 1 h	7.0	0
Photooxidized 4 h	4.6	<15
Cys thioether sulfoxide[c]	0.04	>99

[a] Cyanogen bromide omitted from reaction mixture. [b] Urea omitted from preincubation medium. [c] Prepared by the method of Lederer and Tarin (1971).

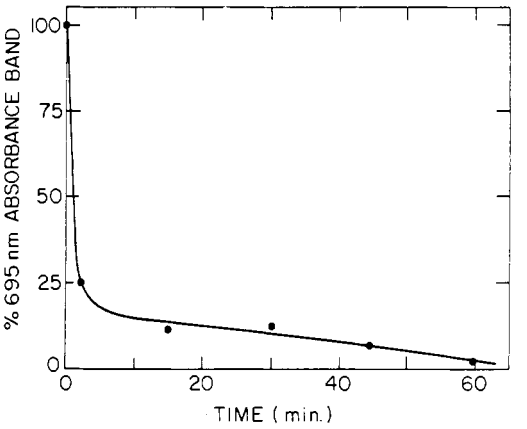

FIGURE 1: The retention of the 695-nm absorbance band of photooxidized ferricytochrome *c* vs. reaction time of methylene blue mediated photooxidation. Cytochrome *c* 110 μM, methylene blue 870 μM, 0-4 °C.

The absorbance spectra of native and methylene blue photooxidized ferricytochrome *c* at pH 7.0 are shown in Figure 2. At this pH, both compounds exhibit spectral characteristics of low spin ferric heme proteins (Falk, 1964). The most notable differences in the spectral properties of the unmodified and photooxidized compound are the increase in the extinction coefficient of the Soret band and the loss of the 695-nm absorbance band in the latter. No new absorbance bands appear between 650 and 800 nm concomitant with the loss of the 695-nm band of the photooxidized sample over the pH range of 4.5–9.0. As the pH is lowered to below 6.5, absorbance bands characteristic of

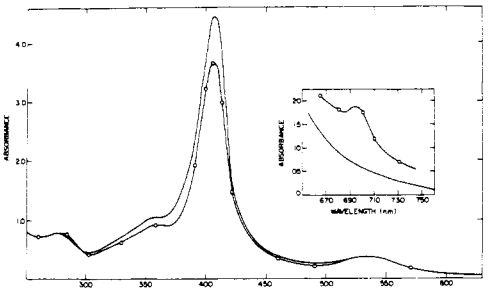

FIGURE 2: Absorbance spectra of ferricytochrome *c* (O) and methionine sulfoxide ferricytochrome *c* (—) in 0.05 M ammonium acetate (pH 7.0). Cytochrome concentration 33 μM, room temperature, 1-cm path length.

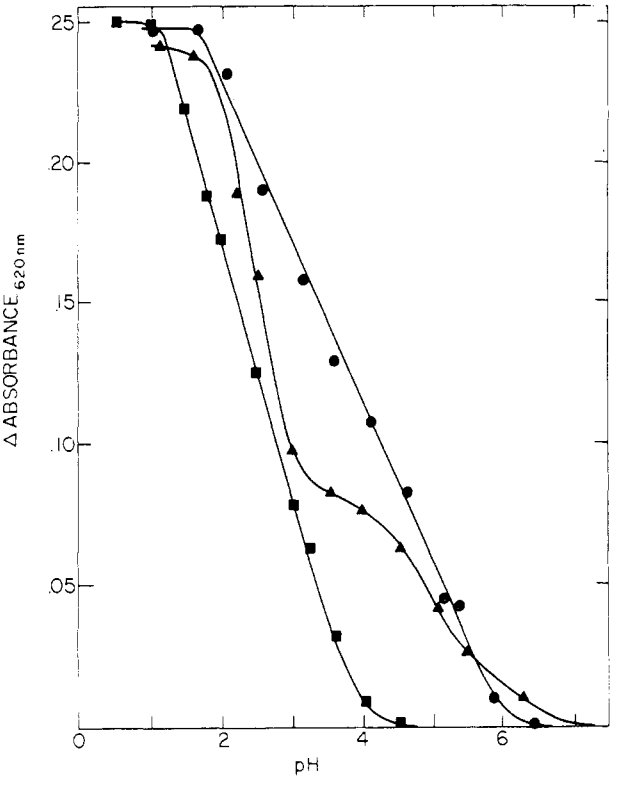

FIGURE 3: Effect of pH on the 620-nm absorbance band of ferricytochrome *c* (■); dicarboxymethylmethionine cytochrome *c* (●); and methionine sulfoxide cytochrome *c* (▲). Cytochrome concentration 100 μM, room temperature.

high spin components (e.g., at 620 nm) appear in the spectrum of this derivative.

The pH dependence of the 620-nm absorbance band of native, dicarboxymethylmethionine, and methionine sulfoxide cytochrome *c* is presented in Figures 3 and 4. Methionine sulfoxide ferricytochrome *c* is converted to high spin form(s) characterized by an absorbance band at 620 nm in a biphasic process with apparent p*K*'s of 2.5 and 5.2. In contrast, the unmodified protein undergoes an apparent monophasic transition with a single apparent p*K* of 2.5.

The circular dichroism spectra of ferricytochrome *c* and methionine sulfoxide ferricytochrome *c* at pH 7.5 are compared in Figures 5–7. There are a number of major differences between the spectra of the photooxidized derivative and those of native cytochrome *c*. The troughs at 690 and 415 nm have been completely eliminated while the peak in the Soret region at 404 nm exhibits a considerable increase in ellipticity. In the intrinsic region there is a slight increase

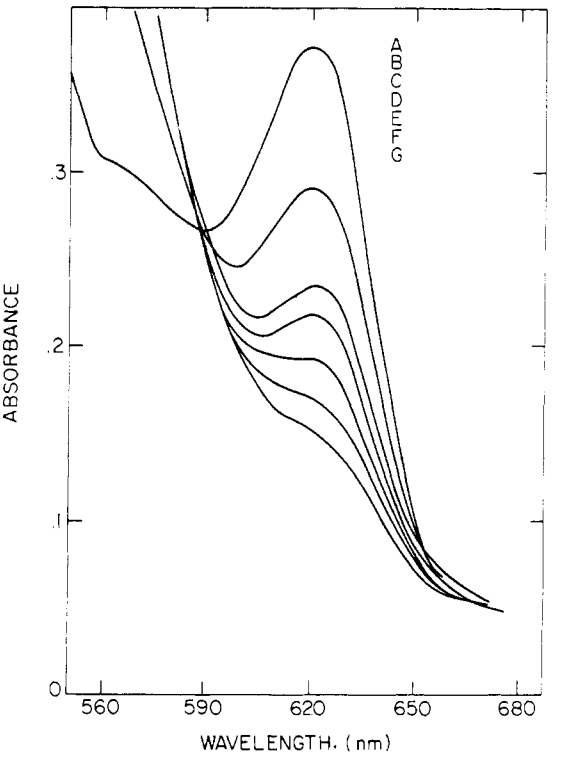

FIGURE 4: The pH dependence of the absorbance spectrum of methionine sulfoxide ferricytochrome *c*. Cytochrome concentration 100 μM, room temperature. (A) pH 1.15; (B) pH 2.51; (C) pH 2.98; (D) pH 3.53; (E) pH 4.51; (F) pH 5.04; (G) pH 5.40.

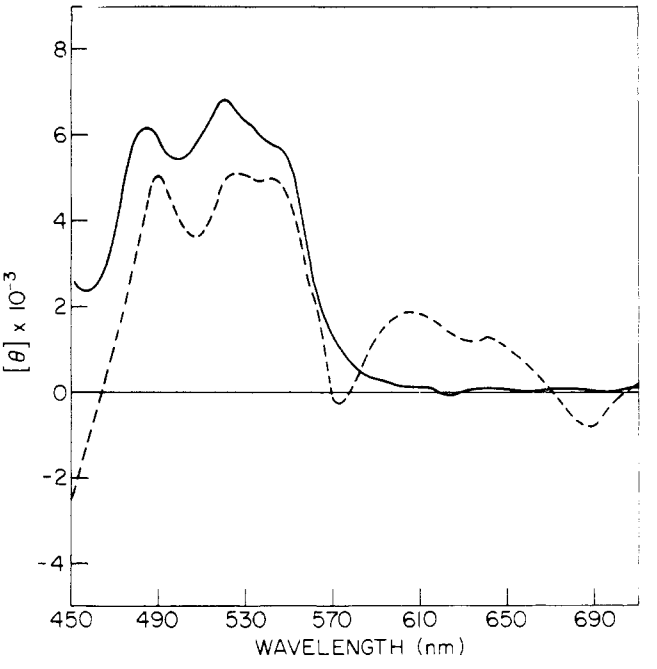

FIGURE 5: Circular dichroism spectra of ferricytochrome *c* (— · —) and methionine sulfoxide ferricytochrome *c* (—) in 0.05 M phosphate buffer (pH 7.5). Cytochrome concentration 135 μM, room temperature, 1-cm path length.

in the ellipticity of the trough at 222 nm. There are only minor further changes in the spectrum of methionine sulfoxide cytochrome *c* at pH 5. At this pH a weak trough appears at 620 nm while the shoulder at 547 nm becomes much more prominent and the peak at 520 nm exhibits a relatively lower ellipticity. The trough at 330 nm is red

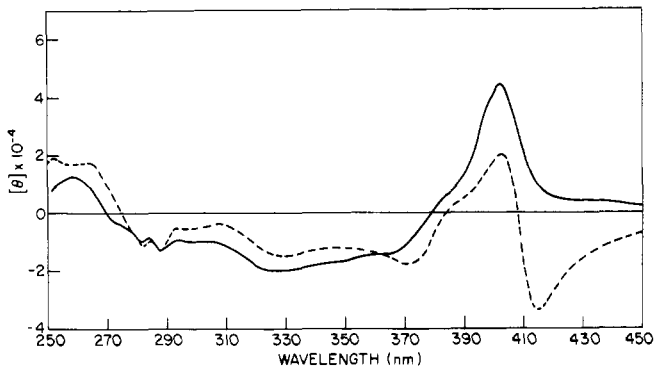

FIGURE 6: Circular dichroism spectra of ferricytochrome *c* (- - -) and methionine sulfoxide ferricytochrome *c* (—), 0.05 M phosphate buffer (pH 7.5). Cytochrome concentration 135 μM, room temperature, 1-mm path length from 450 to 380 nm and 70 μM, 1-cm path length from 380 to 250 nm.

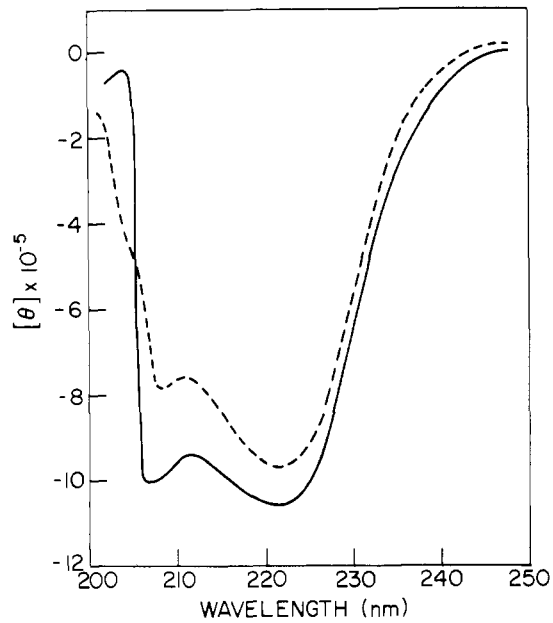

FIGURE 7: Circular dichroism spectra of ferricytochrome *c* (- - -) and methionine sulfoxide ferricytochrome *c* (—), 0.05 M phosphate buffer (pH 7.5). Cytochrome concentration 135 μM, room temperature, 1-mm path length.

shifted to 350 nm. There are no observable changes in the aromatic and intrinsic regions of the spectrum. In Figure 8 the circular dichroism spectra of methionine sulfoxide ferrocytochrome *c* and native ferrocytochrome *c* are compared. Again there are marked changes with a substantial increase in ellipticity and wavelength shift of the Soret band.

Neither treatment with sodium ascorbate or sodium dithionite nor subsequent reaction with cytochrome oxidase resulted in a decrease in the methionine sulfoxide content of the photooxidized protein (Table I) or in the reappearance of the 695-nm absorbance band. Sodium dithionite also failed to reduce L-methionine DL-sulfoxide to L-methionine.

The results of studies of the oxidation of ferrocytochrome *c* and methionine sulfoxide ferrocytochrome *c* are shown in Table V. At pH 7.5 native and methionine sulfoxide ferrocytochromes *c* are oxidized by cytochrome oxidase at identical rates. In both cases, the first-order plots are monophasic, and one first-order rate constant is valid for the entire reaction. At pH 7.5 the rates of autoxidation of unmodified and methionine sulfoxide cytochrome *c* are also similar and are markedly lower than the rates of cytochrome oxidase catalyzed oxidation of these compounds (Table V).

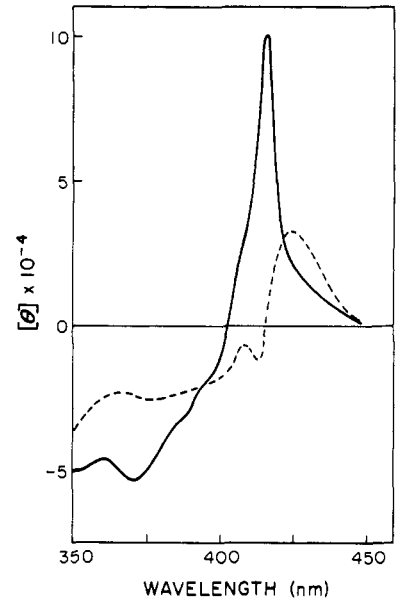

FIGURE 8: Circular dichroism spectra of ferrocytochrome *c* (- - -) and methionine sulfoxide ferrocytochrome *c* (—) in 0.05 M phosphate buffer (pH 7.5) containing small quantities of sodium dithionite. Cytochrome *c* concentration 12.2 μM, methionine sulfoxide cytochrome *c* concentration 12.9 μM, 1-cm path length.

Table V: Rate Constants for the Oxidation of Unmodified and Photooxidized Ferrocytochromes *c*.[a]

Cytochrome *c* Derivative	pH	Concn (M)	First-Order Rate Constant: Autoxidation[b] (sec^{-1})	First-Order Rate Constant: Enzymic[d] (sec^{-1})
Unmodified	5.8	1.3×10^{-5}	*c*	8.4×10^{-3}
Photooxidized	5.8	1.3×10^{-5}	*c*	6.9×10^{-3} [a]
Unmodified	7.5	1.7×10^{-5}	4.0×10^{-5}	$1.02 \pm 0.01 \times 10^{-2}$
Photooxidized	7.5	1.7×10^{-5}	5.3×10^{-5}	$1.00 \pm 0.07 \times 10^{-2}$

[a] Reaction proceeds only to approximately 50% completion at this rate, and then proceeds much more slowly. [b] Initial rate valid for 100 min. [c] Reaction is complex. However, under the conditions of the assay autoxidation was negligible (see Results). [d] Cytochrome oxidase 2.6×10^{-8} M at pH 7.5 and 6.7×10^{-9} M at pH 5.8.

At pH 5.8, the autoxidation of methionine sulfoxide ferrocytochrome *c* becomes much more rapid than at pH 7.5. The reaction is biphasic, the rapid phase (approximately 50%) being completed before termination of the chromatography to remove reductant. The remaining methionine sulfoxide ferrocytochrome *c* is relatively slowly autoxidizable, and for this material the reaction with cytochrome oxidase is biphasic. Approximately half of this material reacts with the oxidase at a rate comparable to that for ferrocytochrome *c* at the same pH while the remainder reacts only very slowly or not at all with the oxidase.

In contrast to its activity with cytochrome oxidase, methionine sulfoxide cytochrome *c* is virtually inactive with succinate-cytochrome *c* reductase. The K_m for the reduction is increased from 4.2 μM for native cytochrome *c*

(Takemori and King, 1964) to approximately 100 μM for the photooxidized derivative. At pH 7.4 at 23 °C the rate of reduction of methionine sulfoxide cytochrome *c* under limiting substrate concentrations was only 3% that of native cytochrome *c*.

The redox potential of methionine sulfoxide cytochrome *c* was determined to be 184 ± 11 mV and was markedly lower than the 260 mV redox potential of native cytochrome *c*.

The interactions of the ferric heme octa- and undecapeptides of cytochrome *c* with 2,2-thiodiethanol, dimethyl sulfoxide, dimethyl sulfone, and *N*-acetylmethionine were studied spectrally at neutral pH and the interactions with *N*-acetylmethionine sulfoxide between pH 4.9 and 7.8. The heme–peptide complex with *N*-acetylmethionine exhibited an absorbance band at 695 nm. None of the other complexes investigated exhibited any spectral absorbance maxima between 650 and 800 nm.

Discussion

Our inability to repeat the reported preparation of methionine-80 sulfoxide cytochrome *c* by photooxidation of cytochrome *c* in the absence of added photosensitizer using the method of Jori et al. (1970) induced us to alter all relevant reaction parameters in the reported procudure (see Methods), particularly since similar attempts to synthesize methionine-80 sulfoxide cytochrome *c* following the procedure of Jori et al. (1970) were also unsuccessful in several other laboratories (see introduction).

Consequently we developed an alternate procedure for specific oxidation of the methionine residues of cytochrome *c* via photooxidation of the protein in the presence of methylene blue. The methionine residues of the protein were shown by amino acid analysis to be the only residues of the protein modified at reaction times of up to 2 h (Tables I and II). The absence of any oxidation of the thioether bridges from cysteine residues at positions 14 and 17 in the primary sequence (Table IV) precludes modifications of these residues from playing a role in the altered redox properties of methionine sulfoxide cytochrome *c*. Presumably the proximity of the thioether bridges to the heme moiety results in quenching of incident radiation by the heme group which effectively protects the bridges from methylene blue mediated photooxidation. It should be noted that under the conditions of the photooxidation experiment (84% acetic acid), methionine-80 is not liganded to the heme iron atom and is therefore susceptible to photooxidation.

In view of the important structural and functional roles proposed for methionine-80 (for a review see Lemberg and Barrett, 1973) it was of particular importance to ascertain the extent of photooxidation of this residue. The results of the cyanogen bromide cleavage and dinitrofluorobenzene treatment which revealed that after 1 h of methylene blue mediated photooxidation, conversion of methionine-80 to the sulfoxide derivative was over 80% complete while the methionine residue at position 65 was approximately 60% modified (Table III) are possibly less accurate than our other criteria. The extent of the loss of the 695-nm absorbance band and the 690-nm circular dichroism trough of methionine sulfoxide cytochrome *c* are probably more sensitive criteria of the extent of photooxidation of methionine-80. The 695-nm absorbance band has clearly been shown to be associated with the methionine-80 sulfur–heme iron bond of cytochrome *c* (Eaton and Hochstrasser, 1967; Schechter and Saludjian, 1967) while the circular dichroism band at 690 nm is probably also associated with this bond. The complete loss of both of these bands from the spectra of methionine sulfoxide cytochrome *c* indicates that photooxidation of methionine-80 is virtually complete in this derivative (Figures 1, 2, and 5). The value of approximately 80% oxidation obtained by chemical analysis within experimental error indicates complete modification of methionine-80. An error of ±15% in the calculated figure arises in large part from the error of ±0.2 residue inherent in conventional amino acid analysis. The homogeneity of methionine sulfoxide cytochrome *c* on Amberlite CG-50 is also in accord with complete modification of methionine-80 in this derivative. The additional modification of methionine residue 65 is expected to be without effect on the structural and functional properties of the protein since carboxymethylation of methionine-65, which results in the introduction of a positive charge at that position, does not appreciably alter the properties of the molecule (Stellwagen, 1968).

The spectral properties of methionine sulfoxide cytochrome *c* were investigated to aid in the identification of the sixth ligand to the heme moiety in this derivative. The virtually unaltered spectrum of methionine sulfoxide cytochrome *c* (Figure 2) relative to that of native cytochrome *c*, especially with regard to the absence of any absorbance band in the region of 620 nm at neutral pH, indicates that this compound, in common with native cytochrome *c*, is in the low spin form (Brill and Williams, 1961). Retention of a low spin configuration by methionine sulfoxide cytochrome *c* indicates that two strong field ligands are bound to the heme iron in the fifth and sixth coordination positions (Falk, 1964). In view of the strength of histidine-18 as the fifth iron ligand in cytochrome *c* and the retention of the p*K* of 2.5 in the spectral titration of methionine sulfoxide cytochrome *c* (Figure 3), it appears certain that histidine-18 is also providing the fifth ligand in this derivative. The nature of the sixth ligand in methionine sulfoxide cytochrome *c* remains to be elucidated. A sensitive index of the coordination of methionine-80 as the sixth ligand in native ferricytochrome *c* is the 695-nm absorbance band. The 695-nm band is lost from the spectrum of ferricytochrome *c* following displacement of methionine-80 as the sixth ligand to the heme by denaturants, extrinsic ligands (Horecker and Kornberg, 1946; Schejter and George, 1964; Sreenathan and Taylor, 1971), or chemical modification of the protein (e.g., Stellwagen, 1968; McGowan and Stellwagen, 1970). Since methionine sulfoxide cytochrome *c* does not exhibit a 695-nm absorbance band it must be concluded that either the electronic nature of the bond is altered or that the sulfur of methionine-80 no longer ligands to the heme iron in this modified form of cytochrome *c*.

It is possible that a strong field ligand such as the ϵ-amino of a lysine residue (e.g., Lys-79) is providing the sixth heme ligand in methionine sulfoxide cytochrome *c*. This is the case with dicarboxymethylmethionine cytochrome *c*, a derivative in which both methionine residues of the protein are carboxymethylated and the sulfur atom of methionine-80 can no longer ligand to the heme iron (Stellwagen, 1968; Keller et al., 1972). In an attempt to determine whether similar liganding occurs in methionine sulfoxide cytochrome *c*, spectral titrations of the two derivatives and of native cytochrome *c* were compared (Figure 3). Low spin–high spin and high spin–high spin transitions of cytochrome *c* are accompanied by corresponding increases in the 620-nm absorbance band (Kaminsky et al., 1972a). Spectral titration of the 620-nm absorbance band of unmodified and

methionine sulfoxide ferricytochromes *c* (Figures 3 and 4) indicates that in acidic solutions methionine sulfoxide cytochrome *c* is characterized by two apparent p*K*'s[2] reflecting the displacement of the fifth and sixth ligands. The first apparent p*K* of 5.5 evidently represents the displacement of the sixth ligand, while the second apparent p*K* of 2.5 corresponds to the displacement of histidine-18 from the central coordination sphere (Kaminsky et al., 1972a). The single apparent p*K* for unmodified cytochrome *c* of 2.5 represents a composite value for displacement of both the fifth and sixth ligands (Babul and Stellwagen, 1972) which would be observed if the two discrete apparent p*K*'s were similar in value or if displacement of the fifth ligand resulted in immediate displacement of the sixth ligand. The spectral titration of methionine sulfoxide cytochrome *c* indicates that the conversion of methionine to methionine sulfoxide at position 80 results in the introduction of a weakened sixth ligand into ferricytochrome *c* which consequently renders the protein more susceptible to acid-mediated displacement of this ligand. The major differences between the titrations of methionine sulfoxide cytochrome *c* and dicarboxymethylmethionine cytochrome *c* (Figure 3) indicate that the sixth ligands to the heme iron differ in the two derivatives which would preclude a lysine residue as the sixth ligand in methionine sulfoxide cytochrome *c* near neutral pH. In addition, the Soret region of the circular dichroism spectrum of methionine sulfoxide cytochrome *c* (Figures 5–7) differs from that of dicarboxymethylmethionine cytochrome *c* (Myer, 1972). In the latter case, although the 415-nm trough is eliminated, the Soret peak has undergone a red shift without the ellipticity increase exhibited by methionine sulfoxide cytochrome *c*.

Methionine-80 sulfoxide thus remains a possible sixth ligand in methionine sulfoxide cytochrome *c*. Model studies of the interaction of sulfur compounds with heme peptides are relevant to attempts to establish the nature of this sixth ligand. It has been reported elsewhere that methionine sulfoxide forms a low spin complex with heme peptides (O'Brien, 1969). However, our observation that the *N*-acetylmethionine sulfoxide–heme peptide complex exhibits mixed spin state but is predominantly high spin suggests that the low spin complex observed by O'Brien (1969) arises from coordination of the α-amino group of methionine sulfoxide (N.B.: O'Brien utilized unblocked amino acids) to the heme peptide. In confirmation, we have demonstrated that glycine, an amino acid in which only the α-amino group can provide a strong field ligand, interacts with heme peptides to produce a characteristic low spin hemochrome spectrum (K. Ivanetich, unpublished results).

Our studies of the interaction of sulfur compounds with heme peptides indicate that the sulfur moiety of *N*-acetylmethionine sulfoxide is a weaker ligand than the thioether sulfur of *N*-acetylmethionine, a fact which is consistent with the weakened sixth ligand bond of cytochrome *c* following oxidation of methionine-80 to methionine-80 sulfoxide. If methionine sulfoxide provides the sixth ligand in methionine sulfoxide cytochrome *c*, it would appear that environmental factors favor a more low spin form rather than the predominantly high spin state seen with *N*-acetylmethionine sulfoxide and heme peptides. Such a ligand bond would probably form via the remaining unshared electron pair on the sulfur atom, since oxygen functional groups generally provide weak field ligands.

Notwithstanding the minor chemical change involved in the modification of cytochrome *c* to methionine sulfoxide cytochrome *c* the conformation of methionine sulfoxide cytochrome *c* in the region of the heme moiety has undergone major disruption. This is clearly shown by the Soret region of the circular dichroism spectrum which is characteristic of transitions involving the heme group and is sensitive to the nature of the fifth and sixth ligands. This region of the spectrum of methionine sulfoxide cytochrome *c* (Figures 5–7) is similar to that of cytochrome *c* in the presence of denaturants (Kaminsky et al., 1972b; Letellier and Schechter, 1973) or at elevated temperatures or low pH (Myer, 1968), especially with regard to the loss of the native trough at 415 nm. The similarities in the circular dichroism spectra of methionine sulfoxide cytochrome *c* and denatured cytochrome *c* further demonstrate that the heme crevice of the derivative is at least partially weakened relative to native cytochrome *c*.

The retention of the two troughs at 282 and 289 nm in the aromatic region of the spectrum of methionine sulfoxide cytochrome *c* implies that the environments of the aromatic residues are unaltered or only slightly altered relative to native cytochrome *c* (Myer, 1972). The circular dichroism spectrum of methionine sulfoxide in the intrinsic region suggests that no major alteration in the conformation of the peptide backbone has occurred. The altered circular dichroism spectrum of methionine sulfoxide ferrocytochrome *c* in the Soret region (Figure 8) indicates that reduction of the heme iron of the modified protein is unable to restore the tightly closed native form of the heme crevice.

Before investigating the function of methionine sulfoxide cytochrome *c* in electron transfer it was necessary to establish that the reductants utilized to reduce the heme iron of this derivative did not also chemically reduce the methionine sulfoxide residues. Consequently, the methionine sulfoxide content of the protein was compared before and after sodium dithionite reduction and subsequent cytochrome oxidase reoxidation of the derivative. The failure of this treatment to alter the methionine sulfoxide content of these preparations (Table I) indicates that none of the observed activity of methionine sulfoxide cytochrome *c* results from the reversion of methionine sulfoxide residues to methionine residues under the conditions of the experiments. This result also tends to mitigate against the proposal of Lardy and Ferguson (1969) that a redox cycling of methionine and methionine sulfoxide at position 80 of cytochrome *c* is involved in mitochondrial oxidative phosphorylation at site III.

Investigations of the activities of methionine sulfoxide cytochrome *c* with solubilized cytochrome oxidase and succinate-cytochrome *c* reductase revealed major differences in the two systems. At neutral pH methionine sulfoxide cytochrome *c* was equivalent to native cytochrome *c* as a substrate for cytochrome oxidase (Table V). At low pH values, however, the reaction of cytochrome oxidase with methionine sulfoxide ferrocytochrome *c* becomes biphasic while enzymatic oxidation of cytochrome *c* remains monophasic (Table V). The decreased activity of a portion of methionine sulfoxide cytochrome *c* present probably reflects the enhanced susceptibility of methionine sulfoxide ferrocytochrome *c* to acid induced spin state changes as seen with the ferric derivative which is partially in the high spin state at

[2] These apparent p*K*'s are not necessarily an actual measure of the p*K* of each ligand. They are composite p*K*'s reflecting all factors that influence the binding of that ligand to the heme, e.g., proximity of a charged residue, hydrogen bonding, and conformational factors.

pH 5.8 (Figure 3). The slow phase of the oxidation reaction probably represents either the slow oxidation of a high spin form of the hemoprotein or the rate-limiting conversion of one or more high spin states to the active low spin form (Table V). Similarly, although methionine sulfoxide and native ferrocytochromes *c* are virtually nonautoxidizable at neutral pH (Table V), the autoxidation of methionine sulfoxide ferrocytochrome *c* becomes biphasic at low pH while the autoxidation of unmodified ferrocytochrome *c* remains monophasic (Boeri and Tosi, 1955; Kaminsky et al., 1971). This difference in oxidizability can be interpreted in terms of the reversible acid mediated spin state changes of methionine sulfoxide cytochrome *c* described above.

In contrast to its activity with cytochrome oxidase, methionine sulfoxide cytochrome *c* is virtually inactive with succinate-cytochrome *c* reductase. In confirmation of these results we have demonstrated that methionine sulfoxide cytochrome *c* does not accept an electron from purified ferrocytochrome c_1 under conditions where electron transfer to cytochrome *c* is very rapid (L. Kaminsky, Y. L. Chiang, and T. E. King, submitted).

The virtual absence of reduction of methionine sulfoxide cytochrome *c* by succinate-cytochrome *c* reductase or by isolated cytochrome c_1 is probably a consequence of the markedly diminished redox potential of the modified protein. The redox potential of 184 mV for methionine sulfoxide cytochrome *c* is below the reported values of 228 mV for cytochrome c_1 in solution (Yu et al., 1973) and 225 mV in the mitochondrion (Wilson et al., 1972). It has previously been suggested that the hydrophobic environment of the heme iron in cytochrome *c* plays a dominant role in maintaining the high redox potential of this heme protein (Kassner, 1972). Consequently an increase in the polarity of this environment would be expected to result in a lowering of the redox potential. The introduction of the polar sulfoxide functional group into the heme crevice and/or the resultant weakening of the heme crevice of cytochrome *c* following the photooxidation of its methionine-80 residue must certainly have increased the polarity of the heme environment and thus lowered the redox potential.

Chemical modifications of specific residues of cytochrome *c* which have resulted in its diminished reducibility by succinate-cytochrome *c* reductase or mitochondria while its oxidizability by cytochrome oxidase was unimpaired have been interpreted to imply the existence of separate binding sites for the reductase and oxidase on cytochrome *c* (e.g., Margoliash et al., 1973). The present studies indicate that such a conclusion is not necessarily correct since the modification may, as a consequence of its induction of conformational changes, be producing a drop in redox potential sufficient to prevent its reduction but which would not necessarily alter its oxidation properties. Similarly, as has recently been suggested by Dickerson and Timkovich (1975), modification of an amino acid residue of cytochrome *c* which results in the markedly diminished ability of the modified cytochrome *c* to be reduced cannot be definitely interpreted to indicate that the modified residue is on the pathway of electrons to the heme iron of cytochrome *c*. It is apparent from the present study that the major role of methionine-80 in the electron transfer properties of cytochrome *c* is to maintain a closed, hydrophobic environment for the heme iron sufficient to provide a redox potential suitable for the biological functioning of cytochrome *c*.

It has been mentioned earlier that Jori et al. (1970) and Folin et al. (1972) have reported on the preparation and properties of a methionine-80 sulfoxide derivative of cytochrome *c* prepared by photooxidation of the heme protein without added photosensitizer. Their product appears to differ from ours only in that methionine-65 is partially oxidized to methionine sulfoxide in our derivative. In view of reports that chemical modification of methionine-65 does not produce changes in structure or function of cytochrome *c* (Stellwagen, 1968) the two products would be expected to exhibit essentially identical properties. Folin et al. (1972) report their product to have an absorbance band at 750 nm and that this band can be reproduced by the addition of *N*-acetylmethionine sulfoxide to a heme peptide. Our modified protein does not exhibit an absorbance band in this region and this band cannot be reproduced in our laboratories or in a number of other laboratories (see introduction), in the manner described. Their spectra are reported at pH 5.5 where the modified cytochrome *c* would be expected to be partially in the high spin state and the sixth ligand partially displaced. Finally, these authors report that their product displays 82% of the activity of native cytochrome *c* in restoring respiration of cytochrome *c* depleted mitochondria and 77% of the activity of native cytochrome *c* with mitochondrial succinate-cytochrome *c* reductase (Folin et al., 1972). Although their activity studies differ from ours only in detail, there are strong contradictions between their results and ours. No explanation can be given for these differences.

In conclusion, we have demonstrated that methylene blue mediated photooxidation of cytochrome *c* results in the conversion of the methionine residues to methionine sulfoxide residues. The resultant molecule is low spin at neutral pH and probably retains methionine-80 sulfoxide as the sixth heme ligand. The molecule is conformationally altered in the region of the heme crevice but is nonautoxidizable. Methionine sulfoxide cytochrome *c* is fully active with cytochrome oxidase but virtually inactive with succinate-cytochrome *c* reductase.

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