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Conformational and Functional Studies of Chemically Modified Cytochrome *c*: Nitrated and Iodinated Cytochromes *c*†

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ABSTRACT: The purification of iodinated (E. B. McGowan and E. Stellwagen (1970), *Biochemistry 9,* 3047) and of nitrated (M. Sokolovsky et al. (1970), *Biochemistry 9,* 5113) cytochromes *c* resulted in the recovery from the former preparation of diiodotityrosyl-cytochrome *c* (DIDT-) with modification of Tyr-67 and Tyr-74, and, from the latter, a mononitromonotyrosyl-cytochrome *c* (MNMT-), with modification of Tyr-67, and mononitrodityrosyl-cytochrome *c* (MNDT-), with the added modification of Tyr-48. The three purified preparations were conformationally characterized using pH-spectroscopy, circular dichroism, thermal denaturation, reducibility with ascorbate, autoxidation with molecular oxygen, and binding with CO. These results are related to the two aspects of biological function, reducibility, measured by NADH-cytochrome *c* reductase, and oxidizability, with cytochrome *c* oxidase, as well as to structure-function relationships in the protein. MNMT-cytochrome *c* was found to be, structurally and conformationally, a single isomer, reducible with ascorbate, with a small, but definite affinity for both oxidation with molecular oxygen and binding of CO. Conformationally, in both valence states of the metal atom, it represents a molecular form with native-like conformation with small but definite perturbations in the immediate vicinity of the heme group, reflected by the destabilization of the Met-80-S-Fe linkage. MNMT-ferricytochrome *c* exhibits a pK of 6.2 for the transformation of the low-spin, native-like spectral form II containing the 695-nm band to a form lacking the 695-nm band. The isomerization at pK = 6.2, when analyzed in terms of the isomerization of the native protein with a pK of 9.2 and the nature of the group involved, indicates that Tyr-67 is not involved in the isomerization of the modified preparation, and possibly not in the native protein as well. In terms of biological function, the partial derangement of reducibility (24%) and the unaltered oxidizability point to the functional significance of Tyr-67, and provide another example of selectivity between the two aspects of physiological function, in agreement with the two-function, two-path operational model of the protein. The MNDT- and DIDT-ferricytochromes *c* exhibited physicochemical properties indicative of gross derangement of both the conformation of the protein as well as of the coordination configuration of the metal atom. The complete inability to accept an electron from NADH-cytochrome *c* reductase in both cases, and the retention of 50% of the oxidizability property of DIDT-cytochrome *c*, were interpreted to be the result of conformational derangement, rather than the added modification of Tyr-48 or of Tyr-74.

The functional role of the tyrosyl side chains in horse heart cytochrome *c* has been studied by different laboratories through the modification of functional groups by a variety of group-specific reagents. Acetylation results in modification of two of the four tyrosyl side chains with concurrent alteration of the stimulation of oxygen uptake in depleted mitochondrial preparations (Ulmer, 1966; Cronin and Harbury, 1965). The nitration with tetranitromethane has been shown to result in preferential modification of Tyr-67, although Tyr-48 (Skov et al, 1969) and/or Tyr-74 are also altered (Schejter and Sokolovsky, 1969; Sokolovsky et al., 1970). Iodination, on the other hand, yields a preparation with the modification of Tyr-67 and Tyr-74 (McGowan and

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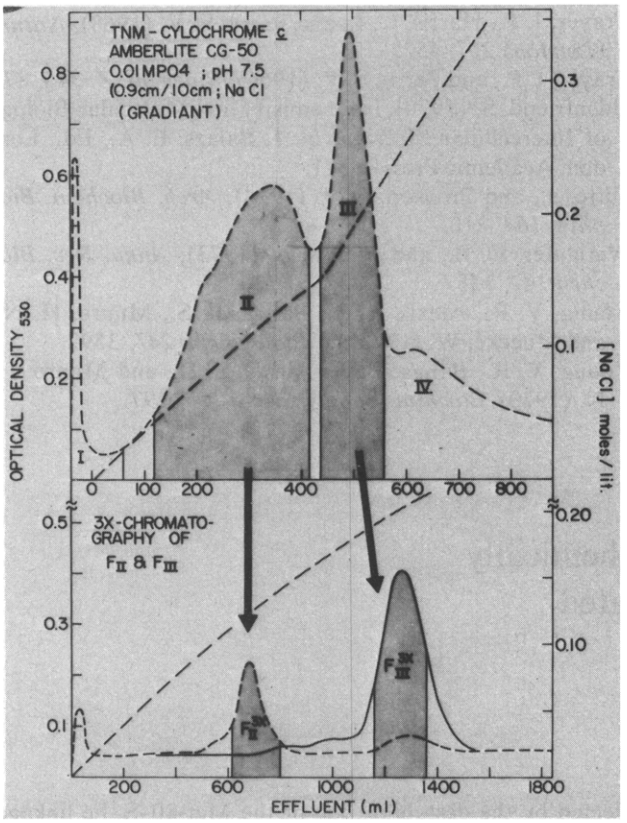

FIGURE 1: Elution patterns of tetranitromethane-treated horse heart ferricytochrome *c* and of three times chromatographed fraction II and III components from Amberlite CG-50. (Top) Nitrated cytochrome *c*; (bottom) three times chromatographed F_{II} and F_{III} components.

Stellwagen, 1970). Nitro-cytochrome *c*[1] was found to be incapable of restoring the respiratory function (Sokolovsky et al., 1970) and the iodinated preparation, when tested for its reducibility with NADH–cytochrome *c* reductase, was shown to retain only 40% of its activity (McGowan and Stellwagen, 1970).

The chemical modification of a protein can result in derangement of its biological function because of the alteration of the functional group directly involved in the catalytic reaction, or because of conformational perturbations resulting from alterations in the primary structure of the molecule. However, for a clear and unambiguous interpretation of the results in terms of either structure–biological function or structure–conformation interrelationships, or both, it is necessary that the systems be devoid of structural complexities, the ideal situation being a single-site structurally homologous modification thoroughly characterized in terms of conformation. Although both the iodinated and the nitrated cytochrome *c* preparations were well characterized, either as such (McGowan and Stellwagen, 1970; Skov et al., 1969) or in a partially purified form (Schejter and Sokolovsky, 1969; Sokolovsky et al., 1970), the physicochemical studies show that the two systems are neither structurally homogeneous (McGowan and Stellwagen, 1970; Schejter et al., 1970) nor are they conformationally well characterized. In addition, in view of recent findings that there is a definite selectivity between the oxidation and reduction functions (Myer, 1972a; Smith et al., 1973; O'Hern et al., 1975), the reported works are limited to either the investigations of the overall effectiveness of the molecule, as in the case of nitro-cytochrome *c* (Sokolovsky et al., 1970), or to the reducibility function of the molecule (McGowan and Stellwagen, 1970). Because of these considerations, we undertook the reinvestigation of both nitrated cytochrome *c* and iodinated cytochrome *c* after appropriate purification in the hope of obtaining structurally homogeneous preparations; this report is the result.

Materials and Methods

Monomeric cytochrome *c*, isolated from a Sigma (type III) preparation, was used for the production of both the nitrated and iodinated cytochromes *c*. The nitration of ferricytochrome *c* was performed by essentially the same procedure used by Schejter and Sokolovsky (1969), and the iodination was conducted according to the procedure described by McGowan and Stellwagen (1970).

The purification of the chemically modified preparations was performed by ion-exchange chromatography using Amberlite CG-50, 200–300 mesh, prepared in the manner already described by Margoliash and Walasek (1967). The elution was performed with a nonlinear NaCl gradient. The fractions containing materials of interest were pooled, desalted by elution chromatography on Sephadex G-25 equilibrated with 1% formic acid by volume, and lyophilized.

The cyanogen bromide fragmentation of the preprations and separation of the fragments were performed according to a procedure already described (Myer, 1972a); the chemical characterization was performed primarily by amino acid analysis using a self-constructed, single-column amino acid analyzer (Bio-Rad Aminex 6 resin), with three buffer elution system. The estimation of methionine sulfoxide was conducted using the indirect procedure (Hirs, 1956), *i.e.*, carboxymethylation at pH 3.0, followed by performic acid oxidation and estimation of methionine sulfone. The tryptophan content was determined by the spectroscopic method already stated, using *N*-bromosuccinimide as the reagent (Myer, 1972a; O'Hern et al., 1975).

The spectroscopic, pH, circular dichroism, and thermal denaturation measurements, etc., were performed according to procedures earlier set down (Myer, 1968a, 1972a,b; Myer and Pal, 1972). Solutions used in these experiments were made in doubly distilled water and freed from oxygen by bubbling ultrapure nitrogen for a period of at least 20–30 min.

The reducibility of the preparations was measured spectrophotometrically at 550 nm using Sigma NADH–cytochrome *c* reductase as the electron donor system. Essential details of the experimental conditions were the same as those of McGowan and Stellwagen (1970) and as described in the earlier publication (Myer, 1972a). The oxidizability property was also measured spectrophotometrically at the same wavelength using cytochrome *c* oxidase as the acceptor according to a procedure described elsewhere (O'Hern et al., 1975). Cytochrome *c* oxidase used in these investigations was a gift from Professor W. Coughey, Colorado State University, Fort Collins, Colo. The reduction of the preparation for oxidizability measurements was performed either

[1] Abbreviations used are: nitro-cytochrome *c*, the product of horse heart ferricytochrome *c* after nitration with tetranitromethane and chromatography on Amberlite CG-50 (fraction II) (Schejter and Sokolovsky, 1969; Sokolovsky et al., 1970); MNMT-cytochrome *c*, nitrated cytochrome *c* fraction with modification of Tyr-67; MNDT-cytochrome *c*, nitrated cytochrome *c* fraction with modification of two tyrosyl side chains, Tyr-67 and Tyr-48; DIDT-cytochrome *c*, purified fraction I component from iodinated cytochrome *c* with modification of Tyr-67 and Tyr-74, both as diiodo derivatives.

Table I: Amino Acid Composition[a] of Native, Nitrated, and Iodinated Preparations and of Various Fractions Obtained by Chromatographic Purification.

Amino Acid	Native Protein	Nitrated Preparation[b]	Cytochromes *c*			Iodinated-Cytochromes *c*		
			F_{III}^{3X}	F_{II}^{3X}	F_I	Preparation[c]	F_I^{2X}	F_{II}
Asp	8.1 (8)	8.0	8.1	7.9	7.9	8.1	8.0	8.2
Methionine sulfoxide[d]	0.1 (0)	0.0	0.1	0.0	ND	0.3	0.1	ND
Thr	9.5 (10)	9.4	9.2	9.4	9.3	9.5	9.1	8.7
Glu	12.2 (12)	12.8	11.9	12.2	12.4	11.8	12.0	11.7
Gly	11.9 (12)	11.7	12.1	11.5	11.8	11.8	11.7	12.5
Ala	5.9 (6)	5.9	6.0	5.8	5.9	5.7	6.1	6.2
Half-Cys	1.7 (2)	1.7	1.8	1.8	1.6	1.7	1.6	1.7
Val	3.1 (3)	3.0	3.0	2.9	3.0	3.0	2.9	2.9
Met	1.8 (2)	1.7	1.8	1.6	1.7	1.6	1.6	1.5
Ile	5.8 (6)	5.9	6.0	6.0	5.9	5.9	5.8	5.9
Leu	6.1 (6)	6.2	5.9	6.2	5.8	5.8	5.7	5.9
Tyr	3.8 (4)	2.6	2.8	1.9	1.5	1.7	1.7	2.3
Phe	3.9 (4)	4.0	4.0	3.7	3.9	3.9	3.8	3.9
Mononitrotyrosine[e]	0.0 (0)	1.2	0.7	1.3				
His	2.9 (3)	2.9	2.9	3.0	2.9	2.9	3.0	2.7
Lys	19.4 (19)	18.7	19.3	19.5	18.8	19.2	19.4	19.5
Arg	1.9 (2)	1.7	2.1	1.9	1.8	1.8	1.9	1.8
Diiodotyrosine[f]						1.5	2.0	0.8
Trp[g]	0.9 (1)	ND	0.9	0.8	ND	ND	0.9	ND

[a] Average of estimations based on Asp = 8; Glu = 12; Val = 3, and His = 3. [b] Preparation with 40 *M* excess of tetranitromethane. [c] Preparation with 16 *M* excess of KI_3 at pH 7.0 in 0.5 *M* phosphate buffer. Reaction time 20 min. [d] Estimated indirectly as methionine sulfone (Myer, 1972a). [e] Based on color constant of phenylalanine. [f] Estimated spectrophotometrically (McGowan and Stellwagen, 1970). [g] Estimated spectrophotometrically from ΔOD_{280} resulting from treatment with *N*-bromosuccinimide (NBS) according to a procedure already described (see Results).

by addition of ascorbate followed by exclusion chromatography or by addition of solid sodium borohydride and appropriate pH adjustment of the solution. Both of these procedures were used for the native and the iodinated preparations, whereas reduction with ascorbate followed by exclusion chromatography was the only procedure employed for the nitrated cytochromes. In order to determine the V_{max}, measurements were continued at higher protein concentrations (20–50 μmol/l.) and estimated by conventional $v_0/(S)_0$ vs. v_0 plots. The activities are expressed as a percentage of the native protein to facilitate the comprehension of the various data in relation to one another.

The concentrations of the modified preparations as well as of the purified components were determined by using the estimated extinctions at the 528-nm peak in the ferric form of the samples based on pyridine hemochrome with a molar extinction of 30.1×10^3 at 550 nm. The following were the extinctions used for each of the purified preparations: MNMT-ferricytochrome *c*, $9.8 \times 10^3\ M^{-1}$; MNDT-ferricytochrome *c*, $9.1 \times 10^3\ M^{-1}$; and DIDT-ferricytochrome *c*, $9.7 \times 10^3\ M^{-1}$.

Results

Purification and Chemical Characterization: Nitrated Cytochrome c. The chromatographic purification of nitrated cytochrome *c* on Amberlite CG-50 yields four distinct components (Figure 1). The amino acid analysis of the fraction IV component showed it to be the native protein. The apparent difference in the amino acid compositions of fractions II and III from that of the native protein is in their tyrosyl contents. On the average, a loss of a single residue is apparent in the fraction III component, and a loss of two tyrosyl residues in the fraction II component (Table I). The fraction I component exhibits the loss of more than two tyrosyl residues and was found to be heterogeneous on further chromatography at pH 6.5.

During the rechromatographic purification of the nitrated cytochrome *c* fractions, there always remained an appreciable amount of material tightly bound to the top of the column. When the tightly bound material was recovered with 0.6 *M* NaCl solution and subjected to rechromatography after appropriate desalting, etc., an elution pattern like that of the starting material was usually the result, but again with a substantial loss due to tightly bound material at the top. It was observed that if the step involving lyophilization for the recovery of solid material is eliminated, and the flash evaporation step for the concentration of the pooled fractions is restricted so as not to yield solutions with concentrations higher than 10^{-4} *M*, the resulting solutions exhibit little or no tendency to form the tightly bound substances. The purified fractions were stored in solution form as eluates from the Amberlite CG-50 column. These solutions were transferred to the appropriate solvent systems by exchange chromatography on Sephadex G-25.

Iodinated Cytochrome c. The chromatography of iodinated cytochrome *c* under conditions similar to those used for the purification of nitrated cytochrome *c* (Figure 1) yielded three distinct components at NaCl concentrations of 0.12, 0.2, and 0.24 *M*/l., respectively. The component eluting at 0.24 *M* NaCl concentration was found to be the unreacted protein. The component eluting at 0.12 *M* NaCl concentration, the major fraction, about 60–70% of the material, exhibited the loss of two tyrosyl residues (Table I), while the analysis of the minor component eluting at a salt concentration of 0.2 *M* showed an average reduction of 1.7 residues/protein molecule. Since only the fraction I component exhibited a composition representing, on the average, modification of a whole residue of the protein, this component was subjected to further purification.

The iodination of proteins results in the formation of both the mono- and the diiodinated derivatives of the tyrosyl residues (McGowan and Stellwagen, 1970). The spectroscopic estimation of the diiodinated tyrosyls based on changes of absorbance at 310 nm in the pH range 4.5–8.5,

using an ϵ of 22.4 nM^{-1} cm^{-1} (McGowan and Stellwagen, 1970) gave a value of 2.0 ± 0.2 diiodinated tyrosyls/protein for the fraction I component, and a value of 0.8 ± 0.2 (Table I) for the fraction II component. Fraction I therefore contains two diiodinated tyrosyl residues. In view of the observation that only two tyrosyls are apparently lost (Table I), fraction I iodinated cytochrome *c* was taken to be a structurally homogeneous species with modification of two tyrosyl residues, both to the extent of diiodination (DIDT-cytochrome *c*). On similar bases, the fraction II component of iodinated cytochrome *c* must be considered a heterogeneous mixture of structurally different molecular forms. Therefore any further consideration of this modified form is not included in the report.

Chemical Characterization. Iodination of cytochrome *c* under the conditions used in these studies has been shown to result in modification of Tyr-67 and Tyr-74, and both are preferentially iodinated to the extent of diiodination under these conditions (McGowan and Stellwagen, 1970). Since DIDT-cytochrome *c* amounts to 60–70% of the total protein, since only two tyrosyl residues/protein molecule are lost (Table I), and since the spectroscopic analysis showed the modifications to be the diiodination (see preceding section), the apparent locations of the modified residues must be the two susceptible groups in the protein, Tyr-67 and Tyr-74 (McGowan and Stellwagen, 1970). No further attempts were made to characterize this preparation.

The nitration of horse heart cytochrome *c*, under the experimental conditions used in these studies, and based on the chemical characterization of the partially purified preparation, has been shown to result in the preferential modification of Tyr-67 with the additional alteration of Tyr-48 (Skov et al., 1969; Sokolovsky et al., 1970): the major product of the modification to be the 3-nitro derivative of the tyrosyl side chains. The amino acid composition of fractions II and III reflects the loss of two and one tyrosyl residues, and the estimated amounts of modified tyrosines were found to be 1.3 and 0.7 residues/protein (Table I). Although the sum of the modified and the unmodified tyrosines is appreciably lower[2] than the expected total of 4 residues/protein, the observation that the ratio of the estimated amounts of modified tyrosines in the two fractions i.e., about 2:1, is similar to that obtained from estimations of modified tyrosines on the basis of the recovered unaltered tyrosines (Table I), leaves little doubt as to the extent of modification in the two components, i.e., two tyrosines and one tyrosine from fraction II and III components, respectively. Regarding the nature of the modification of the tyrosines, the position of the unknown in the amino acid profile was identical with that of 3-nitrotyrosine, between phenylalanine and histidine. Since there were no detectable amounts of other modified forms in the elution profile,[3] especially any corresponding to 3,5-dinitrotyrosine,[4] the nature of the modification was taken to be the formation of 3-nitrotyrosine, which is consistent with the characterization reported earlier (Skov et al., 1969; Sokolovsky et al., 1966).

As to the location of the modified amino acids, the characterization on the basis of cyanogen bromide fragmentation showed that both preparations contain a single nitrated tyrosyl residue in the fragment of 66–80, which contains two tyrosyl residues, 67 and 74 (Margoliash and Schejter, 1966). Since the characterizations of the nitro-cytochrome *c* thus far undertaken (Skov et al., 1969; Schejter and Sokolovsky, 1969; Sokolovsky et al., 1970) show that of the two tyrosines in the fragment 66–80 Tyr-67 is preferentially modified, and since there is minimal or no indication of the susceptibility of Tyr-74 to nitration under the experimental conditions used (Skov et al., 1969), it is appropriate to designate position 67 as the nitrated site in both fractions III and II. In the case of the fraction II component, an additional mononitrated tyrosyl residue was found in the fragment from the sequence 1 through 65, which contains the second susceptible tyrosyl residue of the protein, residue 48 (Skov et al., 1969). Thus in terms of the chemical characterization of the two fractions of nitrated cytochrome *c*, fraction III represents a molecular form with modification of a single tyrosyl side chain, Tyr-67, and fraction II is the modified form with the additional modification of Tyr-48; the modification in both instances is the formation of the mononitrotyrosyl derivative. The two modified forms are therefore referred to as mononitromonotyrosyl- (MNMT-) and mononitrodityrosyl- (MNDT-) cytochromes, respectively.

Spectroscopic Properties: Absorption Spectra. The absorption spectroscopic characteristics of MNMT-, MNDT- and DIDT-cytochromes *c* and of the native protein, corresponding to the pH's of each of the four different spectral types, are compared in Table II, which also includes the values of the apparent p*K*'s for each of the transitions. The coordination configuration of the metal atom consistent with the pH–spectroscopic behavior of various modified preparations is schematically presented in Figure 2. All preparations at low pH's exhibit typical high-spin spectral type I similar to that of the native protein under identical conditions. Likewise, all preparations at high pH, type IV, exhibit spectral characteristics identical with those of the native protein. The spectral identity at both extremes of the pH range for the three preparations and the native protein implies that the integrity of the heme group is maintained during both the iodination and the nitration reactions of the protein.

The pH–spectroscopic behavior of all three preparations in the range 2–10 is distinct from that of the native protein and from each other. The high-spin type of MNMT-cytochrome *c* reverts to a native-like low-spin type II with a p*K* of 2.8. It exhibits the characteristic 695-nm band, and lacks the features usually ascribed to the high-spin form of heme iron, in particular the 620-nm band (Day et al., 1967). The absolute extinction of the 695-nm band, $|\epsilon|_{695}$, if estimated according to the procedure described by Kaminsky et al. (1973), is, however, only 122 M^{-1} at pH 4.5 in comparison

[2] The low yield of modified tyrosines could be due to a combination of reasons: (i) the color constant used was that of the closest amino acid in the elution profile, phenylalanine, which could be different from that of the modified tyrosines, (ii) nitrotyrosine was destroyed during preparation of the samples, or (iii) a combination of both the preceding. The lower yield of nitrated tyrosines is not unique to this protein, but seems to be a common feature of systems subjected to nitration, e.g., elastase, cytochrome *c* peroxidase, and horseradish peroxidase (Skov et al., 1969).

[3] In addition to the unknown identified as 3-nitrotyrosine, the elution profiles of the fraction II components exhibited an additional unknown between mononitrotyrosine and phenylalanine. Since the relative amount of this component was small, 10–15% of mononitrotyrosine, and since the peak followed the break point of the third buffer, no estimation was made.

[4] The position of 3,5-dinitrotyrosine has been reported to be between valine and methionine (Sokolovsky et al., 1966). Attempts to detect the presence of any unknown in this region, in spite of expansion of the elution pattern by lowering the pH of the second buffer from 4.2 to 3.9, were negative.

Table II: Spectral Characteristics of Native and Various Modified Ferricytochromes *c* at Different pH's Corresponding to the Four Spectral Types.

pH:	<2		4–5		7–8		>10
Type:	I		II		III		IV
			Absorption Maxima (Shoulder); Italicized Minima (nm)				
			Native Ferricytochrome *c*				
	618, 570, (530), 495, 394	p*K* = 2.6	695, (565), 528, 409, *365*		695, (565), 528, 408, *365*	p*K* = 9.2	(560), 528 406, *365*
			Diiododityrosyl-Ferricytochrome *c* (DIDT-)				
	620, (570), (530), 495, 395	3.0	620, (565), 528, 404, *365*	5.8	(565), 528, 407, *365*		(565), 528 406, *365*
			Mononitromonotyrosyl-Ferricytochrome *c* (MNMT-)				
	218, 570, (530) 497, 395	2.8	695, (565), 528 406, *365*	6.2	(565), 528 407, *365*		(562), 528 406, *365*
			Mononitrodityrosyl-Ferricytochrome *c* (MNDT-)				
	620, 570, (530), 497, 392	3.2	620, (565), 528, 402, *365*		[(618), (565), 528, 405, *365*]	8.2	(565), 528, 407, *365*

to 198 M^{-1} observed for the native protein. MNMT-cytochrome *c* type II further reverts to a low-spin form (type III) lacking the 695-nm band with an apparent p*K* of 6.2, which finally changes to spectral type IV at extreme alkaline pH's. The spectroscopic changes encountered during the transition of type II to III, if viewed in terms of the disappearance of the 695-nm band, which has been associated with the integrity of the Met-80–S–Fe linkage of the protein (Schechter and Saludjian, 1967), are comparable to those observed for the native protein with an apparent p*K* of about 9.2. The resulting spectrum after completion of the transition from spectral type II to III is, however, different from that of the native protein at alkaline pH (Table II), especially with respect to the position of the Soret band and the extinctions of both the Soret and the 528-nm bands. Since the common denominator between the type II → III transition of MNMT-cytochrome *c* and type III → IV of the native protein is the elimination of the 695-nm band, indicating the disruption of the Met-80–S–Fe linkage (Schechter and Saludjian, 1967), the transition with a p*K* of 6.2 of MNMT-cytochrome *c* thus reflects basically the same perturbation of the native protein at alkaline pH's.

The pH transformation of spectral type I to type IV of MNDT-cytochrome *c*, however, occurs through two distinct steps with the formation of a single distinct intermediate. The first is centered around a pH of 3.2, and the second, at a pH of about 8.2. The intermediate form, possibly type II, exhibits features of both the high- and the low-spin heme systems, e.g., the concurrent presence of the 620-nm band, a characteristic of the high-spin form (Day et al., 1967), and the 528-nm band and the Soret band above 400 nm, characteristic of low-spin heme iron systems. Spectroscopic characteristics similar to those of spectroscopic type II of MNDT-cytochrome *c* have also been observed in simple heme *c* systems (deaminated heme octapeptide and heme octapeptide in the presence of 8 *M* urea) (Myer and Harbury, 1973; Y. P. Myer and H. A. Harbury, unpublished data), as well as in other modified preparations of the protein (O'Hern et al., 1975; Myer and Pal, 1972; Schejter and Aviram, 1970). The mixed-spin spectral intermediate then reverts to a native-like low-spin spectral form with a p*K* of about 8.2. This final transition is very broad and in general reflects the complexities of a system with multiple components; i.e., lack of clearly defined isosbestic points.

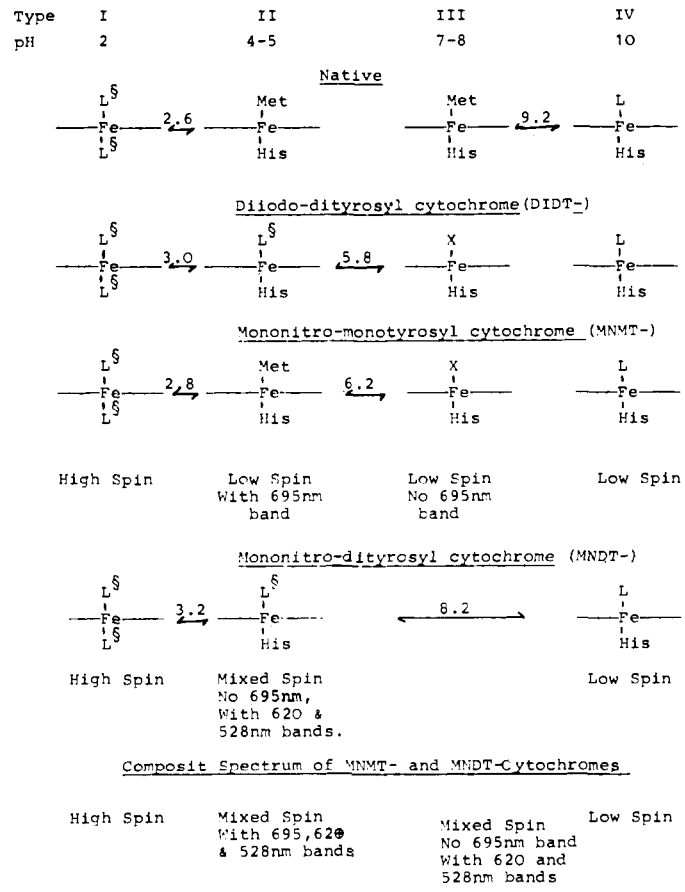

FIGURE 2: Schematic representation of the coordination configuration of heme iron compatible with the pH–spectroscopic behavior of native and various modified preparations of horse heart ferricytochrome *c*. Included are the observed p*K*'s for each transition, the expected spectral characteristics of each purified component, and the composite of two components of nitrocytochrome *c*. L and X stand for two different, but strong, ligand-field groups of the protein, and $L^{\S}$ stands for a weak ligand-field functional group.

In contrast to the spectroscopic behavior of MNDT-ferricytochrome *c*, the pH behavior of DIDT-cytochrome *c* is typical of the modified proteins, yielding four distinct spectral forms (Table II). High-spin spectral type I changes to a mixed-spin spectral type II with a p*K* of about 3, which fur-

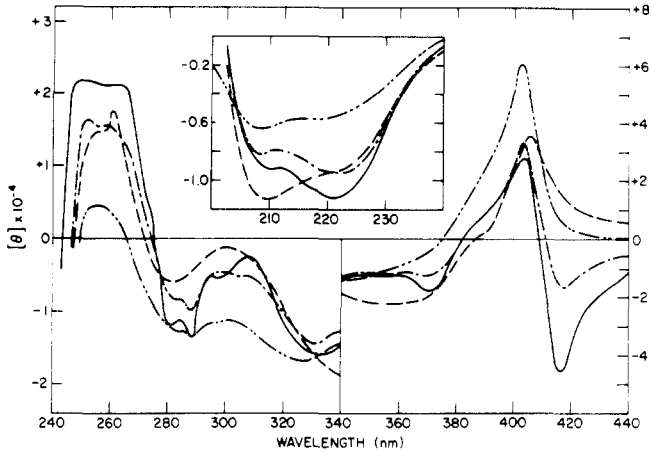

FIGURE 3: Circular dichroism spectra of native and various modified cytochrome *c* preparations in the ferric state of the metal atom; pH 7.0, 0.1 *M* phosphate buffer. (—) Native cytochrome *c*; (— —) MNMT-cytochrome *c*; (- - -) MNDT-cytochrome *c*; (- · · -) DIDT-cytochrome *c*.

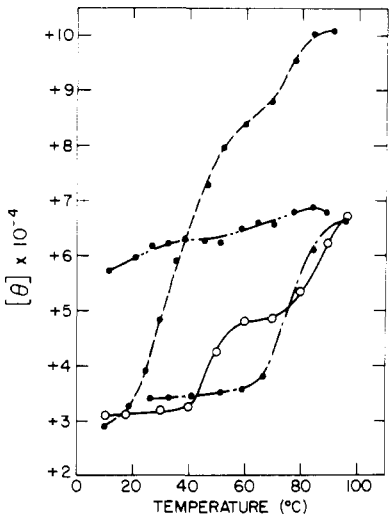

FIGURE 4: Thermal denaturation profiles of native cytochrome *c* and modified preparations in the ferric state of the metal atom; pH 7.0, in water. (—) Native cytochrome *c*; (- - -) MNMT-cytochrome *c*; (- - - -) MNDT-cytochrome *c*; (- -) DIDT-cytochrome *c*. Ellipticity measured at the 404-nm Soret peak.

ther undergoes transformation to a typical, low-spin type III with a p*K* of 5.8, and finally goes to a native-like, low-spin type IV at extreme alkaline pH's. In this case, neither of the types in the pH range 2–10 exhibits the native Met-80–S–Fe-linked 695-nm band.

Circular Dichroism Spectra. In Figure 3 are compared the CD spectra of the various modified preparations and of the native protein in the ferric state of the metal atom at pH 7.0. The replacement of the doubly inflected Soret CD spectrum by a single positive band for both MNDT- and DIDT-ferricytochromes *c*, with the concurrent simplification of the dichroic pattern in the δ-absorption region and the loss of resolution of the aromatic dichroic inflections at 281 and 289 nm, are characteristics of the conformationally and configurationally deranged native protein (Myer, 1968b). The differences in the intrinsic dichroic patterns, especially as seen in DIDT-ferricytochrome *c* (Figure 3), are obviously those of a protein with a partially deranged polypeptide conformation (Myer, 1968b). In terms of the

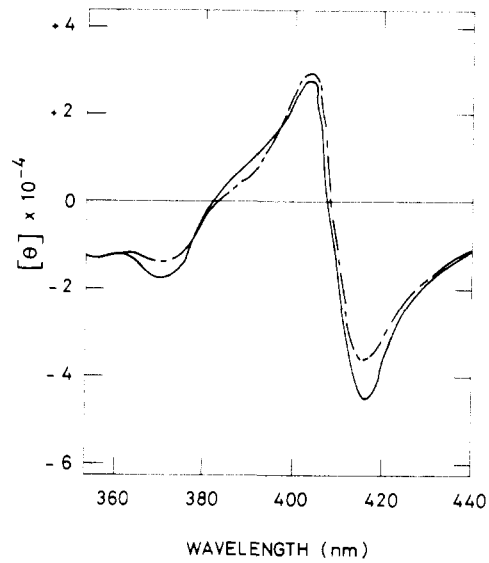

FIGURE 5: Soret circular dichroism spectra of native and mononitromonotyrosyl-ferricytochromes *c* at pH 4.5. (—) Native cytochrome *c*; (- - -) MNMT-cytochrome *c*.

conformation of the molecule, both MNDT- and DIDT-ferricytochromes *c* at neutral pH's therefore represent molecular forms with a deranged coordination configuration of the metal atom as well as an altered polypeptide conformation of the molecule. The differences in the dichroic curves of the two preparations, both in the Soret and other regions of the spectrum (Figure 3), however, do indicate that the two preparations are conformationally distinct from one another. The difference in their susceptibilities to thermal denaturation (Figure 4), as well as the spectroscopic differences (Table II), are consistent with the above inference regarding the relative conformational states of the two preparations. The apparent difference between MNDT- and DIDT-ferricytochromes *c* is not surprising, since the two preparations differ not only in the nature of the substituents, mononitro vs. diiodo, but also in one of the two modified tyrosyl side chains, i.e., the internally hydrogen bonded Tyr-48 (Takano et al., 1973) in MNDT-cytochrome *c* and the surface Tyr-74 in the DIDT preparation.

The dichroic spectrum of MNMT-ferricytochrome *c* is in many details like that of the native protein (Figures 3 and 5). The Soret spectrum maintains its complexity, although to a reduced degree, and the dichroic details in the aromatic region are similar to those of the native protein. The slight reduction in ellipticity of the Soret negative band, with the concurrent enhancement of the positive peak, with a parallel reduction of dichroic absorption in the near-uv region, as well as the reduced ellipticity of both the 222- and 208-nm bands in the intrinsic region, are typical of the variations observed for the first denaturation step of the native protein (Myer, 1968b). These variations of the CD spectrum have been correlated with the uncoupling of porphyrin–polypeptide interactions with little or no unfolding of the polypeptide conformation of the molecule (Myer, 1968b), which apparently is the conformational state of ferric MNMT-cytochrome *c* at neutral pH. The absence of the first denaturation step in the melting curves with only a slight lowering of the melting temperature of the second step (Figure 4) does support the above inference regarding its conformation. Similarly, the lowered stability of the Met-80–S–Fe linkage, i.e., the shift of the p*K* from about 9.2 to 6.2 for its

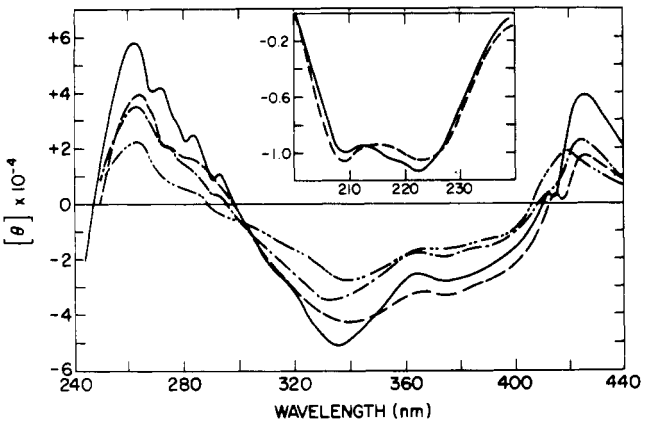

FIGURE 6: Circular dichroism spectra of native cytochrome *c* and various modified preparations in the ferrous state of the metal atom. Key same as in Figure 4.

disruption (Table II), as well as the spectroscopic differences, are also consistent with the inferred conformational state of this preparation.

The dichroic spectra of both MNMT- and DIDT-cytochromes *c* in the ferrous state of the metal atom are in general like that of the native protein (Figure 6). The spectra of both these preparations maintain almost all the features of the native spectrum, including the shoulder or inflection at about 415 nm. However, the dichroic curves of both of these modified preparations do show an overall decrease in ellipticities of almost all the inflections. Whether this is a result of error in the determination of concentration or whether it is a reflection of the presence of conformational perturbation could not be determined at this time. The fact that both of these preparations exhibit reduced resolution of the peaks in the 280–290-nm region, the region reflecting contributions from the invariant Trp-59 residue (Myer, 1968b), does permit one to state that both of these preparations, even in the reduced state of the metal atom, are conformationally perturbed, especially in the immediate vicinity of the Trp-59 residue. Since Trp-59 is buried deep in the heme crevice (Takano et al., 1973) and its activity results from dipole–dipole interaction (Myer, 1968b), the nature of the conformational perturbation could be ascribed to a weakening of the dipole coupling, i.e., a loosening of the crevice. The chemical properties, such as a well-developed autoxidizability, an affinity for CO binding, and an altered $\epsilon_{550}/\epsilon_{520}$ ratio (Table III), all support the above conclusion regarding the conformational state in both DIDT- and MNMT-ferrocytochromes *c*. It should be pointed out that although the two preparations in the ferric state of the metal atom were found to be conformationally distinct (see preceding section), in the ferrous state the apparent differences are more or less minimal in nature.

The CD spectrum of reduced MNDT-cytochrome *c*, on the other hand, is distinct from both that of the native protein and from those of the MNMT and DIDT derivatives of the protein (Figure 6). Since there are no demonstrably explicit interrelationships between the CD spectrum and the conformation of ferrocytochrome *c*, as in the case for ferricytochrome *c* (Myer, 1968b), it is rather difficult to interpret the dichroic differences in the reduced form of the preparation in terms of specific conformational details. However, it is not unreasonable to state that MNDT-ferrocytochrome *c* is conformationally distinct from any of the systems investigated and is a conformationally deranged form of the molecule.

Table III: Physicochemical Properties and Activity of Native and Various Modified Preparations of Horse Heart Cytochrome *c* at Neutral pH.

Property	Native Protein	Preparation: MNMT-Cytochrome *c*	MNDT-Cytochrome *c*	DIDT-Cytochrome *c*
Spectra				
Ferric				
Soret (nm)	408	407	405	407
$\lvert\epsilon\rvert_{695}$ (M)	166	56	NP	NP
Ferrous				
Soret (nm)	416	417	ND	418
ϵ_{soret} (mM)	138	167	ND	152
ϵ_{550} (mM)	27.4	26.5		25.1
$\epsilon_{550}/\epsilon_{520}$	1.71	1.53	1.01	1.56
Reducibility				
Ascorbate	Yes	Yes	None	None
NADH–cytochrome *c* reductase (%)[a]	100	24	<10	<10
Oxidizability				
Oxygen	None	Slow	Rapid	Rapid
Cytochrome *c* oxidase				
Pseudo-first-order cons. (%)[b]	100	82	ND	28
V_{max} (%)[c]	100	105	ND	56

[a] Determined according to procedure already described (Myer, 1972a). [b] Determined according to the procedure already described (O'Hern et al., 1975). [c] Determined by conventional $v_0/(S)_0$ vs. v_0 plot. Concentration range for these investigations was 10–50 μmol/l.

Oxidoreduction Properties and Enzymatic Activity. The results of studies of oxidation–reduction reactions, and measurements of reducibility by NADH–cytochrome *c* reductase and oxidizability by cytochrome *c* oxidase for each modified preparation and the native protein are summarized in Table III. MNMT-cytochrome *c* exhibited reducibility with ascorbate at neutral pH's and the resulting spectrum had a ratio of 1.53 for $\epsilon_{550}/\epsilon_{520}$. (The rate of reduction, however, was slower than that of the native protein under the same conditions.) The addition of ascorbate to solutions containing MNDT-ferricytochrome *c*, on the other hand, resulted in a spectrum characterized by the presence of small, distinct peaks at both 550 and 520 nm, a typical hemochrome spectrum, but with an extinction ratio $\epsilon_{550}/\epsilon_{520}$ of only 1.01. The magnitude of spectral changes at 550 nm, when compared to those observed upon reduction of MNMT-cytochrome *c*, reflect a change of only 10–15%, which, if taken at face value, can be interpreted as a reflection of the partial reducibility of the preparation. Since reduced MNDT-cytochrome *c* exhibits a rather high degree of autoxidizability with molecular oxygen, the partial reduction may be a reflection of the presence of dissolved oxygen in the system. Since the reduction was performed with a 1000-fold excess of ascorbate, since the solutions were freed of oxygen by bubbling ultrapure nitrogen, and since the experiment was performed under anaerobic conditions, the possibility of interference by dissolved oxygen seems highly unlikely. When the reduction was performed at alkaline pH (pH 10), the resulting spectrum was very similar to that of the native protein, reflecting the complete reducibility of type IV of this preparation. Since MNDT-cytochrome *c* exhibits a broad transition from type II at acid pH's to the

type IV alkaline form (Table II; Figure 2), the 10–15% reducibility at neutral pH's, if estimated on the relative magnitude of ϵ_{550}, with the concomitant presence of the oxidized form, could easily be due to properties of both types II and IV forms of MNDT-cytochrome *c*. Complete ascorbate reduction at alkaline pH and almost no ascorbate reduction at pH 6.0 is indeed consistent with the foregoing suggestion. The stable form of MNDT-cytochrome *c* at neutral pH's therefore is not reducible by ascorbate. Attempts to reduce DIDT-ferricytochrome *c* with ascorbate at neutral pH's were also of no avail.

All three preparations, whether reduced with ascorbate or with dithionite, where applicable, exhibited well-developed affinities for both autoxidation with molecular oxygen and for CO binding. Of the three preparations, reduced MNMT-cytochrome *c* showed the least tendency to react with CO and molecular oxygen.[5] The development of accessibility of heme iron to extrinsic ligands is a clear indication of a loosened heme crevice of the molecule. However, the relative slowness of autoxidation and formation of the CO complex in the case of MNMT-cytochrome *c* suggests that the perturbations of the heme environment in this modified preparation are relatively small as compared to those of the other two preparations.

In terms of cytochrome *c* activity, oxidizability, and reducibility with physiological donors and acceptors, all three modified preparations exhibit a deranged acceptance of electrons from NADH–cytochrome *c* reductase. The derangement is almost complete in the case of MNDT- and DIDT-cytochromes *c*, whereas MNMT-cytochrome *c* maintains as much as 24% of the native reducibility property (Table III). As to their capabilities to donate an electron to cytochrome *c* oxidase, MNMT-ferrocytochrome *c* retains the capability intact, whereas the DIDT preparation exhibits a definitely reduced capability when compared to the native protein, i.e., 56% in terms of the V_{max} and 28% in terms of V_{max}/K_m.

The measurement of the oxidizability property of MNDT-cytochrome *c* was prevented as the preparation exhibited little or no reducibility with ascorbate, and reduction with dithionite is not suitable in this case, because it results in structural alteration of the molecule, i.e., of the nitrotyrosyl derivatives to aminotyrosyl derivatives (Sokolovsky et al., 1967).

Discussion

Horse heart cytochrome *c* contains four tyrosyl residues at positions 48, 67, 74, and 97. The X-ray diffraction data show that, of the four tyrosyl side chains, only Tyr-97 is completely exposed. Tyr-67 and Tyr-48 are buried deep in the heme crevice and are involved in internal hydrogen bonding with Thr-98 and one of the two propionic acid side chains, respectively (Takano et al., 1973). Tyr-74 is located on the surface and lies flat, which renders it only partially accessible to solvent systems. Iodination has been shown to result in modification of both Tyr-67 and Tyr-74 (McGowan and Stellwagen, 1970), while nitration has been shown to result in preferential modification of Tyr-67 (Sokolovsky et al., 1970) with the additional modification of Tyr-48 (Skov et al., 1969) and/or of residue 74 (Sokolovsky et al., 1970). The recovery of a single major component from iodinated cytochrome *c* with modification of two residues, both to the extent of diiodination, confirms the earlier suggestion regarding the nature of the product. The isolation of a mononitromonotyrosyl derivative with modification of Tyr-67 and of another component with modification of both Tyr-67 and Tyr-48 in general supports earlier inferences regarding the behavior of the protein toward nitration with tetranitromethane (Skov et al., 1969; Sokolovsky et al., 1970).

The spectroscopic (Table II) and oxidation–reduction linked properties (Table III) of DIDT-cytochrome *c* are very similar to those of the unpurified preparation (McGowan and Stellwagen, 1970). The earlier inference that iodination of Tyr-67 and Tyr-74 results in disruption of the central coordination configuration, with loosening of the heme crevice, is thus borne out. Although these CD studies generally support the foregoing conclusion, they also indicate the existence of definite derangement in the polypeptide conformation of the molecule. The altered intrinsic CD spectrum and the drastically altered stability of the preparation to thermal denaturation (Figures 3 and 4) are clear indications of the existence of gross conformational alteration of the molecule. Thus the iodination of Tyr-67 and Tyr-74 results in not only the loosening of the heme crevice and disruption of the central coordination complex, but also of the organized polypeptide conformation of the protein. Even in the reduced form, the physicochemical properties (Table III) including the dichroic spectrum manifest the existence of definite perturbation, especially in the immediate vicinity of the heme group. Whether this represents an uncoupling of the heme–polypeptide interactions or an altered coordination configuration or both could not be presently discerned from these measurements. The observation that the dichroic spectrum of reduced DIDT-cytochrome *c* is apparently very similar to that of the native protein, however, does permit one to state that if the molecule maintains these perturbations in the reduced state of the metal atom, the magnitude of such perturbations must be small so as not to be clearly observable in the dichroic spectrum of the protein.

A similar conclusion regarding the conformational status of MNDT-ferricytochrome *c* is also apparent, although in terms of the nature and the magnitude of conformational derangement, it is distinct from DIDT-ferricytochrome *c*. The development of a Soret ellipticity comparable to that of the native protein at elevated temperatures (Figure 4), the greatly simplified dichroic spectrum in the near-uv region, changes in the intrinsic absorption region (Figure 3), and the occurrence of only minor changes upon heating (Figure 4) demonstrate that the apparent effect of modification of both the Tyr-67 and Tyr-48 side chains is almost complete derangement of the heme environment with alteration of the coordination complex, as well as alterations in the polypeptide conformation of the molecule. The grossly simplified dichroic spectrum in the reduced state of the metal atom (Figure 6), and the well-developed autoxidizability and affinity for CO binding (Table III), are indicative of a degraded conformation of the molecule in the ferrous state of the metal atom. The spectroscopic behavior, especially the lack of the 695-nm band in the pH range 4–9 (Table

[5] Cassell and Fridovich (1975) have recently concluded from studies of autoxidation of ferrocytochrome *c* that the apparent lack of autoxidizability of the native protein is due to the reduction of ferric cytochrome *c* by the superoxide radical, rather than the limited accessibility of ferrocytochrome *c* to molecular oxygen. The higher level of autoxidizability of the MNDT- and DIDT-cytochromes *c* and the slight enhancement of autoxidizability in MNMT-cytochrome *c* could be a reflection of the conformational perturbation of the ferric forms of the molecules, rather than of the ferrous forms of the preparations, since the ferric forms are the ones which exhibit maximal conformational perturbation, except in the case of DIDT-cytochrome *c*.

II), as well as identity in the pH-spectroscopic behavior (Figure 2), are in agreement with the above conformational and configurational interpretations of MNDT-ferricytochrome *c*.

In contrast to both DIDT- and MNDT-ferricytochromes *c*, MNMT-ferricytochrome *c* maintains most of the conformational characteristics of the native protein. Spectroscopically and conformationally it represents a molecular form with native-like conformation except for small but genuine perturbation of the heme environment, which is characterized by uncoupling of the heme-polypeptide interactions. The lowering of the p*K* by about three units, 6.2 instead of 9.2, for the transition involving the disruption of the Met-80-S-Fe linkage (Table II), the lack of the first step in the thermal denaturation profile (Figure 4), and the partial simplification of the Soret CD spectrum with little, if any, alteration in other regions of the spectrum (Figure 3) are all indicative of the apparent effect of the alteration of Tyr-67 in MNMT-cytochrome *c*, the slight loosening of the heme crevice. In the reduced state, the altered $\epsilon_{550}/\epsilon_{520}$ ratio, and the slight development of both autoxidizability and CO binding tendencies (Table III), are also indicative of small, but finite, perturbation of the electronic configuration of the heme group, even though differences suggestive of an altered conformation failed to show up in the dichroic spectrum (Figure 6). Thus conformationally and configurationally MNMT-cytochrome *c* represents a molecular form with small perturbations in the immediate vicinity of the heme group, the observable effect of which is an increased accessibility of iron to extrinsic ligands in the reduced state, and lowered stability of the central coordination complex in the ferric forms of the molecule. MNMT-cytochrome *c* is conformationally and configurationally much like NBS-modified cytochrome *c* form I, which contains the modification of Trp-59 (Myer, 1972a,b; Myer and Pal, 1972; O'Hern et al., 1975). Since both Trp-59 and Tyr-67 lie deep in the heme crevice, since both of these functional groups are involved in internal hydrogen bonding (Takano et al., 1973), and since the position of the substituents in both cases is at a position other than that involved in the hydrogen bonding, the ortho position to the phenolic -OH in the case of nitrated cytochrome *c*, and to the nitrogen of the indole moiety in the case of the NBS derivative, the similarity in the conformational perturbation is not too surprising.

A comparison of the physicochemical properties of MNMT- and MNDT-cytochromes *c* with those of nitro-cytochrome *c* (Sokolovsky et al., 1970; Schejter et al., 1970), and a comparison of the observed structures of the two purified components with those suggested for the two forms of nitro-cytochrome *c* (Schejter et al., 1970) reflect inconsistencies. The chemical, spectral, and oxidation-reduction linked properties, as well as the nature and magnitude of the structural alterations of MNMT-cytochrome *c*, are identical with those proposed for form I of nitro-cytochrome *c* (Schejter et al., 1970). The behavior of MNDT-cytochrome *c*, on the other hand, is very similar to that suggested for the isomer, form II, but structurally both Tyr-67 and Tyr-48 are modified, not just Tyr-67, as has been stipulated for form II. Since the physicochemical properties of nitro-cytochrome *c* including the oxidation-reduction characteristics and the pH-spectroscopic behavior (Tables II and III) can be generated by a mere summation of the properties of MNMT- and MNDT-cytochromes *c* (Schejter et al., 1970, Figure 5 vs. Figure 2, this work), and since the purified components are constituents of the chromatographic fraction previously taken as nitro-cytochrome *c* (Schejter and Sokolovsky, 1970, Figure 1 vs. Figure 1, this work), a simpler explanation of the behavior of nitro-cytochrome *c* on the basis of a mixture of MNMT-cytochrome *c*, containing the modification of Tyr-67, and MNDT-cytochrome *c*, containing the additional modification of Tyr-48, seems more appropriate, rather than an explanation of a mixture of structurally identical, but conformationally different, mononitromonotyrosyl-67 derivatives of the protein as suggested by Schejter et al. (1970). A summation of the properties of MNMT- and MNDT-cytochromes *c* in a ratio of about 4:1 would in general generate the observed physical properties of the nitro-cytochrome *c* of Schejter et al. (1970) (Figure 5 vs. Figure 2 this work), except for the reported higher degree of nonreducibility with ascorbate and the retention of only 16% of the oxidizability function of the molecule. It is shown in this work that the lyophilization of the nitrated preparations, both in the purified or unpurified state, results in the formation of aggregates (see Results). Such an occurrence is known to alter both activity and physical properties of the protein (Margoliash and Lustgarten, 1962). Since the studies reported earlier were of lyophilized samples, the apparent disparity between the observed physicochemical properties for nitro-cytochrome *c* and those resulting from a summation of properties of the two purified components reported in this work could easily be due to the existence of added heterogeneity in the former preparations resulting from the presence of aggregates.

The transformation with a p*K* of 6.2 of spectral type II to type III of MNMT-ferricytochrome *c* reflects spectroscopic alterations which are in most details identical with those observed for the alkaline isomerization of the native protein centered at pH 9.2. Both involve the elimination of the 695-nm band and the generation of typical low-spin forms. The final states in the two cases, however, are distinct, as evidenced by small variations in the absorption characteristics (Table II). Recent kinetic studies of the alkaline isomerization of the native protein show that the process involves a deprotonation step with a p*K* of about 11 followed by slow conformational reorganization of the protein with an equilibrium constant with an apparent p*K* of −1.3 (Czerlinski and Bracokova, 1971; Davis et al., 1974). A p*K* of 10 or higher for the phenolic hydroxyls in proteins is well accepted; furthermore, studies with model phenols showed that a lowering of the p*K* by 3 pH units upon nitration is also an expected occurrence (Schejter et al., 1970). These observations have lead to the suggestion that the ionization with an apparent p*K* of about 6 in MNMT-cytochrome *c* may involve the Tyr-67 residue and may be a counterpart of the isomerization of native ferricytochrome *c* with a p*K* of 9.2 (Schejter et al., 1970). A recent observation that a pH-transition identical in most spectroscopic details with that observed in MNMT-ferricytochrome *c* with a p*K* of 6.2 (i.e., a transition of the native-like type II form to a typical low-spin type III without the 695-nm band) is also present in one of the purified components from *N*-bromosuccinimide-modified cytochrome *c*, NBS form I (O'Hern et al., 1975; Myer and Pal, 1972), in which neither the tyrosyl side chains are modified nor are there any significant alterations of the phenolic p*K*'s, definitely rules out the possibility of the involvement of Tyr-67 in this pH-dependent behavior of this modification. Whether this can be extrapolated to suggest noninvolvement of tyrosyls in the alkaline isomerization of the native protein is debatable, but the recent ki-

netic study of the isomerization of the native protein does point to their noninvolvement (Davis et al., 1974).

Regarding the reason for the pH shift of three units for the disruption of the Met-80–S–Fe linkage in the protein upon the modification of either Tyr-67, as in MNMT-ferricytochrome *c*, or upon modification of Trp-59, NBS form I (O'Hern et al., 1975; Myer and Pal, 1972), the common denominator appears to be the nature and the magnitude of the conformational perturbation of the molecule. The role of conformation in the stabilization of the S–Fe linkage in the protein in the ferric state of the metal atom is apparent from the observation that in model systems (such as heme octapeptide, which lacks the conformational implications of the native protein) this structure is exceedingly unstable, so much so that molar ratios of more than 1000 between *N*-acetylmethionine ester and the model system failed to result in saturation of the Fe–S linkage (Harbury et al., 1965). The weakened heme–polypeptide interaction and the consequent loosening of the heme crevice resulting from the modification of Tyr-67 or Trp-59 could easily result in the lowered p*K* observed for the disruption of the central coordination complex of the molecule. Since the ionization of groups far removed from heme has been known to result in alteration of the metal–ligand bond distances and of the spin-state equilibrium (Smith and Williams, 1970), the closeness of the observed p*K* to that of the noncoordinated histidines in the protein, His-33 and His-26, is noteworthy.

In a consideration of structure–biological function relationships in the protein, the derangement of reducibility and the unaltered oxidizability in the case of MNMT-cytochrome *c*, which contains the modification of a single tyrosyl residue at position 67, leaves little doubt as to the importance of the integrity of Tyr-67 in the oxidoreduction function of the molecule, especially in the reducibility with the physiological electron donor, NADH–cytochrome *c* reductase. This is consistent with the inference of Margoliash et al. (1973), arrived at through independent investigations of a purified fraction of nitrated cytochrome *c* exhibiting physical properties of that of the partially purified nitrated cytochrome *c* already investigated by Schejter et al. (1970). Whether the derangement of reducibility is due to the alteration of the electronic configuration of the phenolic group of Tyr-67 or due to the conformational consequence of this chemical alteration in the interior of the molecule, i.e., the loosening of the heme crevice, or both, could not be discerned from the information thus far available. However, the feasibility of the selective derangement of the reducibility property without any noticeable perturbation of the electron donor function of the molecule does provide another case supporting the independence of the two aspects of the oxidoreduction property of the molecule, i.e., a two-path, two-function model (Myer, 1972a; O'Hern et al., 1975; Margoliash et al., 1973). The further disruption of the reducibility function, as seen in MNDT-cytochrome *c*, as well as the derangement of both the reducibility and the oxidizability functions in the case of DIDT-cytochrome *c*, on the other hand, seem to be the result of gross conformational derangement of the molecule, rather than the blocking of additional functional groups in the protein. The spectroscopic and dichroic characterization substantiates this assessment of the conformational and functional states of both preparations.

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