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Conformational and Functional Studies of Chemically Modified Cytochromes: *N*-Bromosuccinimide- and Formyl-Cytochromes *c*†

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ABSTRACT: *N*-Bromosuccinimide-cytochromes *c* (Myer, Y. P. (1972), *Biochemistry 11,* 4195) and formyl-cytochrome *c* (Aviram, I. and Schejter, A. (1971), *Biochim. Biophys. Acta 229,* 113) have been chromatographically purified, and the resulting components have been characterized in terms of their structure, conformation, and function. The activity measurements are considered in terms of the oxidizability, as the transference of an electron to solubilized cytochrome *c* oxidase, and reducibility, as the tendency to accept an electron from NADH-cytochrome *c* reductase. Conformational characterization has been carried out by absorption measurements, pH-spectroscopic behavior, circular dichroism, thermal denaturation, ionization of phenolic hydroxyls, the tendency to form the CO complex, and autoxidation with molecular oxygen. NBS-cytochrome *c* yields two major components, the relative proportions of which, with increasing modification of the protein, exhibit a pattern typical of the formation of the two in a consecutive manner. The first product contains the modification of the Trp-59 and Met-65 side chains, and the second contains the added modification of Met-80. The former in both valence states of iron is more or less like the native protein, except for an apparently slightly loosened heme crevice; the latter, as in other modifications involving modification of centrally coordinated Met-80, was found to be in a conformational state characteristic of the native protein with a disrupted central coordination complex, a loosened heme crevice, and small, but finite derangement of the polypeptide conformation. Functionally, the first component reflected 55% of the reducibility property and an unimpaired oxidizability property, while the latter exhibited derangement of both aspects of cytochrome *c* activity. Formyl-cytochrome *c* yielded a single component with modification of Trp-59. Conformationally, in both valence states, it is a molecular form with a disrupted central coordination complex, a loosened heme crevice, and gross derangement of the overall protein conformation. It exhibits a minimal reducibility property, 12%, whereas it retains a native-like tendency to transfer an electron to cytochrome *c* oxidase. The data from the NBS-cytochrome *c* components are analyzed with reference to the two forms in the earlier studies of the unpurified preparations. The results are found to be in agreement with one another. The selectivity between the reducibility and the oxidizability exhibited by the first NBS component and formyl-cytochrome *c*, irrespective of significant differences in the conformational and coordinational configurations of the two, has been viewed in light of a two-path, two-function model for oxidoreduction, as well as with reference to conformational and structural requirements for the oxidizability and reducibility properties of the molecule.

Chemical modification of specific functional groups has been one of the principal avenues of approach to the elucidation of structure-biological function relationships and the functioning of enzymes on a molecular level. In the case of cytochrome *c*, the role of the invariant tryptophanyl residue in the oxidoreduction function of the molecule has been investigated by the use of two specific modifications of the functional group, the formylation (Aviram and Schejter, 1971) and the *N*-bromosuccinylation (Stellwagen and Van Rooyan, 1967; Yonetani, 1968). In general, these investigations showed that the modification of the tryptophan resi-

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due results in the disruption of the central coordination complex of the molecule with a concurrent loss of biological activity. However, recent structural, spectroscopic, conformational, and functional studies have indicated that the modification of the protein by NBS[1] involves a multiple-site attack, *i.e.*, of the tryptophanyl, methionyl, and tyrosyl residues. The interpretation of the physicochemical and functional behavior necessitated a suggestion of the consecutive formation of two structurally different modified forms of the protein, form I containing the alteration of Met-65 and Trp-59 and exhibiting native-like spectroscopic properties, with altered reducibility but unaltered oxidizability, and form II containing the additional modification of Met-80, resulting in a conformationally deranged and functionally inactive form (Myer, 1972a,b; Myer and Pal, 1972). These studies lead further to the suggestion of a charge-transfer model for reduction of heme iron through orbital overlaps of surface aromatic residue (now known to be the aromatic moiety of Tyr-74; Morrison *et al.*, 1971) → Trp-59 → Tyr-67 → porphyrin ring. Recently, the availability of the three-dimensional structure for both oxidized and reduced cytochromes *c* (Dickerson *et al.*, 1971; Takano *et al.*, 1973) has not only supported the two-path, two-function model for reduction, but also provides a rationale on the molecular level for the irreversibility of the reducibility path and the feasibility of the charge-transfer model. The previous studies of the NBS-cytochromes *c* were performed on unpurified preparations, and even though formyl-cytochrome *c* contains a single-site modification at Trp-59, the investigation of this preparation was restricted to the overall activity of the molecule, *i.e.*, the measurement of the stimulation of oxygen uptake in a cytochrome *c* depleted mitochrondrial system, with no detailed conformational characterization. For these reasons it was felt that a more extensive conformational characterization and an investigation of activity in terms of oxidation and reduction of highly purified modified preparations are essential for the confirmation of the earlier findings and for elucidation of implications of the conformational-functional *vs.* structural-functional aspects of the oxidoreduction function of the molecule. In this communication we report the results of such studies of both the NBS- and the formyl-cytochromes *c*.

Experimental Section

Materials

The modified preparations were obtained by a procedure already described (Myer, 1972a; Aviram and Schejter, 1971). The purification was performed by nonlinear salt-gradient chromatography on Amberlite CG-50 under the conditions specified in the appropriate figure. The Amberlite resin was prepared according to the procedure described by Margoliash and Walasek (1967). The final material was obtained by desalting on Sephadex G-50 equilibrated with either 0.2 M ammonium bicarbonate or 2% formic acid and lyophilization. All other chemicals were of the best grade available.

Methods

The structural characterization (amino acid analysis, cyanogen bromide fragmentation, and resolution of components, etc.), absorption spectroscopic measurements, circular dichroism measurements, pH-spectroscopic titrations,

[1] Abbreviation used is: NBS, N-bromosuccinimide.

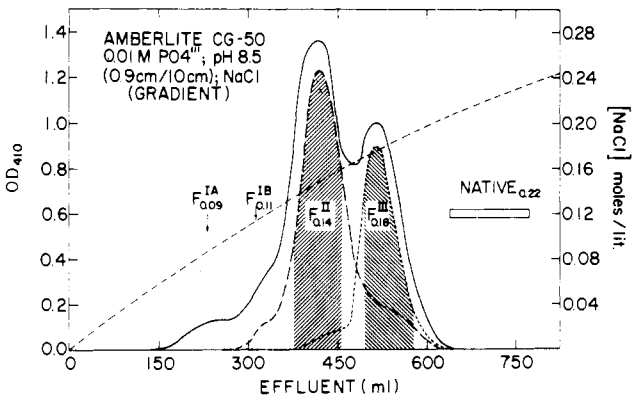

FIGURE 1: Elution pattern of NBS-cytochrome *c* (3.8:1 NBS-cytochrome *c*) and of twice-chromatographed fractions II and III; 100–150 mg of NBS-cytochrome *c* in 0.01 M phosphate buffer (pH 8.5) adsorbed on Amberlite CG-50, sodium form, and eluted with a nonlinear NaCl gradient (constant volume, 2 l. dilute solution of 0.01 M phosphate (pH 8.5) and mixing solution, 0.6 M NaCl in the same buffer). (—) NBS-cytochrome *c*; (– –) rechromatographic pattern of twice-purified fraction II component; (- - -) rechromatographic pattern of twice-purified fraction III component. Shaded areas represent the fractions pooled for final recovery of materials. Rectangular area represents the position of elution of native cytochrome *c*. The dotted line represents the NaCl concentration.

thermal denaturation, measurements of ionization of phenolic hydroxyls, and measurements of reducibility activity with NADH-cytochrome *c* reductase were performed by procedures already described (Myer, 1972a). The oxidizability of the protein was determined as a pseudo-first-order rate constant, *i.e.*, at protein concentrations in which the system obeys pseudo-first-order kinetics, for the oxidation with cytochrome *c* oxidase of borohydride-reduced preparations. The reduction was performed by the addition of small increments of solid sodium borohydride under an atmosphere of oxygen-free nitrogen. The pH of the solution was adjusted to the appropriate value whenever required. A control run, *i.e.*, a run without cytochrome *c* oxidase, was made parallel to each measurement, and results were corrected for any observable autoxidation prior to analysis. The measurements were performed at 550 nm. The details of the run, such as concentration of the components, order of addition, pH, etc., are given in the appropriate figure. The concentrations of the solutions were measured spectroscopically using the following extinctions: native, ϵ_{528} of 10.4 mM; NBS preparations, ϵ_{504}, the isosbestic point (Myer, 1972a), of 6.5 mM; formyl-cytochrome *c*, ϵ_{528} of 9.23 mM.

Results

Purification of Preparations. An example of an elution pattern obtained by Amberlite chromatography of an NBS-cytochrome *c* preparation is shown in Figure 1, which also includes the elution patterns of the third chromatography of the two major components (F^{III} and F^{II}). Figure 2 illustrates the course of events during the NBS reaction in terms of the formation of various molecular species. The pattern in Figure 2 is typical of a system undergoing a sequential reaction with the F^{III} component as a stable intermediate. In addition, there were two more minor components. Appreciable amounts were present only in preparations obtained at 6 or higher molar ratios of the reagent. Upon chromatographic purification under the same conditions, formyl-cytochrome *c* yielded a single component eluting during the early stages of development. Since repeated chromatography failed to show any indication of

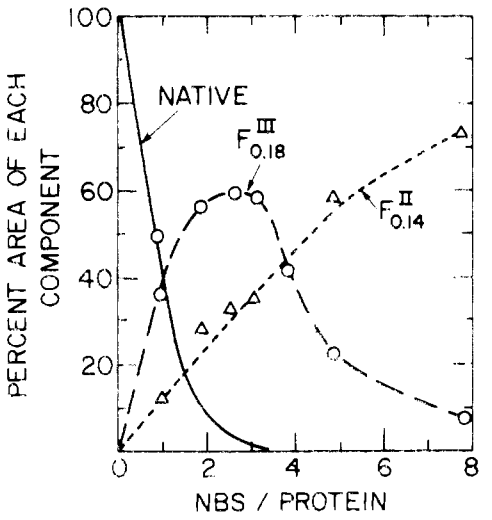

FIGURE 2: Relationship between proportions of unreacted cytochrome *c*; fraction II and III components of NBS-cytochrome *c* in preparations at increasing NBS to protein ratios. The proportion of each component, including that of the native protein, determined by per cent area of the skewed Gaussian curves generating the elution pattern. The fitting of the elution pattern was performed using a Du Pont 310 curve resolver.

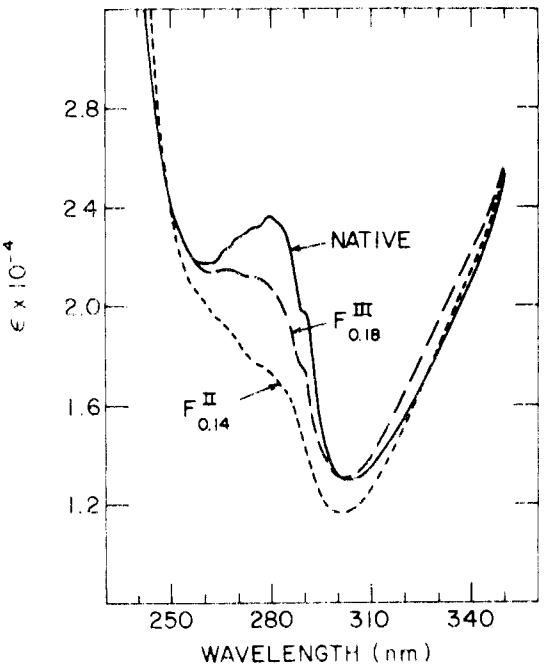

FIGURE 3: Aromatic absorption spectra of ferric cytochrome *c*, and fractions II and III of NBS-cytochrome *c*; 0.05 M phosphate buffer, pH 7.0.

separation or improvement, once-chromatographed preparations were considered purified and used for further studies.

The amino acid analysis of the purifed fractions shows that the apparent modification in the NBS F^{III} component is of the single tryptophanyl and one of the two methionyl side chains; the NBS F^{II} component contains the added modification of the second of the methionyl side chains; and purified formyl-cytochrome *c* contains the modification of the tryptophanyl residue only (Table I). The modification of the methionyl side chains is to the corresponding methionyl sulfoxides. As to which of the two methionines are modified in the NBS F^{III} component, the cleavage of the

Table I: Amino Acid Content of Native and Chemically Modified Cytochromes *c*.

Amino Acid	Native	NBS F^{III} Component	NBS F^{II} Component	Formyl-Cytochrome *c*
Tryptophan[c]	1.0	0.05	0.00	0.05[b]
Methionine sulfoxide[c]	0.0 (1.6)[d]	0.8	1.6	0.1
Tyrosine	3.7	3.6	3.7	3.5
Histidine	3.0	2.8	2.8	2.9

[a] Estimated by spectroscopic procedure using the expression $OD_{280} \times 1.3/5500$ from NBS treatment according to Patchornik *et al.* (1960). [b] Estimated by amino acid analysis after hydrolysis in the presence of thioglycol. [c] Determined indirectly as methionine sulfone after carboxymethylation and performic acid oxidation (Myer, 1972a). Native under identical conditions taken as zero. [d] Methionine sulfone content of native protein after performic acid oxidation.

preparation with cyanogen bromide and characterization of the products showed it to be Met-65. This is indicated from the observation that the NBS F^{III} component upon cyanogen bromide treatment yielded exclusively a fragment resulting from breakage at position 80–81, and not at 65–66, which is expected in the case of the native protein. Since the NBS F^{III} component does not cleave at postion 65–66, Met-65 must be the site of modification, which is consistent with the well-established fact that the presence of methionine sulfoxides prohibits cleavage by cyanogen bromide (Jori *et al.*, 1970). As to the nature of the alteration of the tryptophanyl moieties in both the formyl and the NBS preparations, the former has been shown to be the 1-formylindole derivative (Aviram and Schejter, 1971), whereas the latter is possibly the 5-bromo-2-oxindole derivative (Green and Witkop, 1962; Myer, 1972a). The aromatic absorption characteristics of the NBS components (Figure 3) are consistent with the above structural assignments to the NBS-tryptophan derivatives, and, likewise, the absorption properties of the formyl derivative in the aromatic absorption region (Aviram and Schejter, 1971; not shown here) agree with the suggested nature of the modification in this preparation, as does the observation that treatment at alkaline pH's reverted the molecule into the native form.

Absorption Spectra. The pH-spectroscopic behavior of the native protein and the three modified preparations as reflected by absorption changes in the spin-state-sensitive bands, the 528- and 620-nm bands, and the ligand-sensitive bands, the 365- and 695-nm bands (Y. P. Myer, unpublished data; Shechter and Saludjian, 1967), is shown in Figure 4. In Table II are listed the absorption characteristics of each system corresponding to the various stable spectral types. Unlike the native protein, the NBS F^{II} component and formyl-cytochrome *c* revert from a typical high-spin form at acid pH's to a low-spin form through a well-resolved, two-step transition with apparent p*K*'s of 2.8 and 5.3 and of 3.5 and 5.9, respectively. In the case of the NBS F^{II} component, both steps are seen through changes in the ligand-sensitive (365 nm) and the spin-state-sensitive bands (528- and 620-nm bands; the latter is not shown). The two steps of the transformation in the formyl derivative are not

Table II: Spectral Bands of Native and Various Modified Cytochromes 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	695(565), 528, 409, *365*	695(565), 528, 408, *365*	(560), 528 406, *365*
NBS F^{III} Ferricytochrome *c* Component			
620, 565, (530), 495, 398	695(565), 528, 407, *365*	*695*(565), 528, 406, *365*	(560), 528, 405, *365*
NBS F^{II} Ferricytochrome *c* Component			
620, 565, (530), 495, 398	620(565), 528, 400(365)	(565), 528 406, *365*	530, 405, *365*
Formyl–Ferricytochrome *c*			
620, (530), 495, 394	620(565), 530, 401(362)	(565), 530 406, *365*	530, 405, *365*

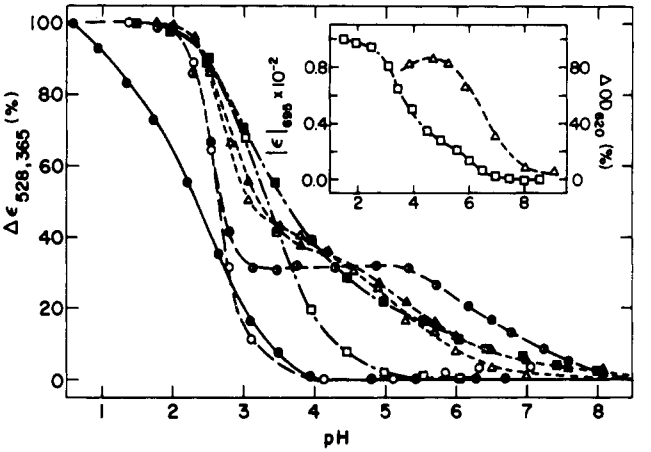

FIGURE 4: Spectroscopic–pH titration curves of the native protein and of modified preparations at various spectral bands in the ferric state of the metal atom. Conditions: in water; pH adjusted with HCl. (—) Native cytochrome *c*; (- -) NBS fraction III component; (- - -) NBS fraction II component; (O—O) formyl-cytochrome *c*. (●, O, △, and □) at 528 nm; (◒, ▲, ■) at 365 nm. Inset: (△ — △) changes in $|\epsilon|_{695}$ as a function of pH for the NBS F^{III} component; (□ — □) changes at the 620-nm band as a function of pH for formyl-cytochrome *c*.

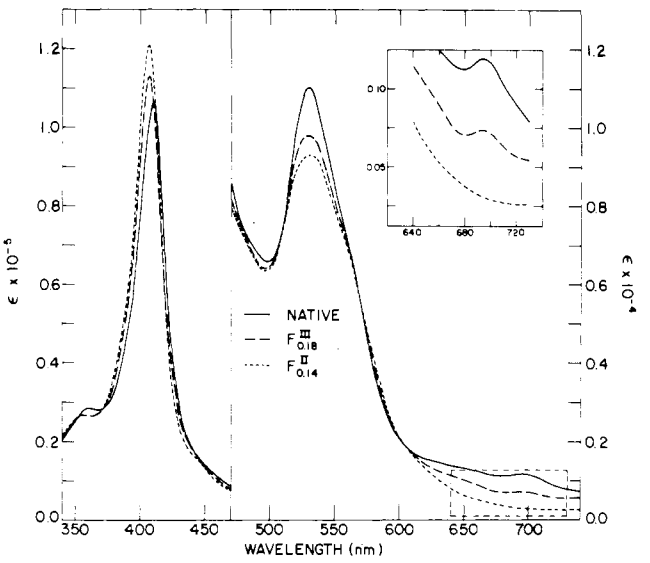

FIGURE 5: Absorption spectra of native cytochrome *c* and of the NBS F^{III} and F^{II} components in the ferric state of metal atom; pH 7.0, 0.1 M phosphate buffer. (—) Native cytochrome *c*; (- -) NBS F^{III} component; (- - -) NBS F^{II} component.

apparent in alteration at the 528-nm band, but are clearly reflected in the corresponding spin-state-sensitive 620-nm band (see Figure 4, inset). The intermediate spectral form in the two cases exhibits features characteristic of both low- and high-spin forms of the protein, and the low-spin type III form in the two preparations lacks the 695-nm band characteristic of the Met-80-S-Fe linkage as seen in the native protein. The transformation of the high-spin acidic form to the low-spin neutral pH form without the 695-nm band through a two-step transition discerned by changes of both the spin-state and the ligand-sensitive bands is a feature common to all cytochrome *c* systems containing an altered central coordination complex configuration as a result of chemical modification of the Met-80 side chain, as in the carboxymethylated cytochromes *c* (MacDonald, 1974). This is indeed the situation in the NBS F^{II} component. The absence of the 695-nm band in the spectrum of low-spin formyl-cytochrome *c* is also a clear indication that, although Met-80 is not modified in this preparation, it has been displaced from a coordinating position. The low-spin forms at neutral pH's in both these preparations revert further to a low-spin form at alkaline pH's indistinguishable from that of the native protein under the same conditions.

Unlike the pH-spectroscopic behavior of the NBS F^{II} component and formyl-cytochrome *c*, the NBS F^{III} component exhibits a single-step transition if monitored at the 528-nm band; it lacks the spin-sensitive 620-nm band above pH 4; it exhibits a well-developed 695-nm band in the pH range 4–8; and it shows evidence of a two-step transition if monitored at the ligand-specific 365-nm band (Figures 4 and 5). The first transition, generating a low-spin form with the 695-nm band, is centered at a pH of 2.4, and the latter is centered at a pH of 6.5. The alteration with increasing pH of the absolute extinction of the 695-nm band results in a transition comparable to that observed through spectral changes at the 365-nm inflection. Since the spectroscopic features in the pH region 4–5 are characteristic of a typical low-spin molecular form, including the presence of the 695-nm band (Table II), the first transition must be the reflection of the generation of a native-like coordination configuration of the molecule. The second transition, observable through alterations at both 365 and 695 nm, must be a reflection of the replacement of Met-80-S from the central coordination position. A situation comparable to that in the second pH transition also exists in the native protein, but the apparent pK for this replacement is 9.1, instead of 6.5 as observed here. If the transition with a pK of 6.5 in the NBS F^{III} component and the transition with a pK of 9.1 in the native protein represent the same event, then the lowering of the pK by 2.6 units could be taken as a reflection of the destabilization of the coordination configuration of the metal atom as a result of modification in this preparation. Likewise, the observed spectral differences at neutral pH between the native protein and the NBS F^{III} component (Figure 5) must be a consequence of a loosened and/or de-

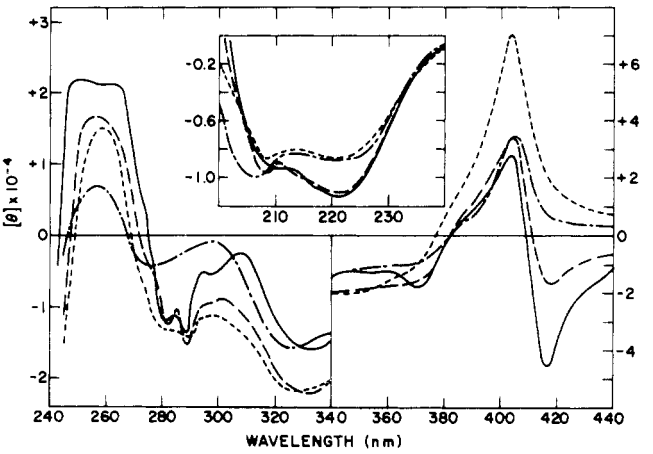

FIGURE 6: Circular dichroism spectra of native cytochrome *c* and modified preparations in the ferric state of metal atom; pH 7.0, 0.1 M phosphate buffer. Dimensions: region 240–440 nm, (deg cm^2)/dmol of protein; Region 200–240 nm, (deg cm^2)/dmol of amino acids). (—) Native cytochrome *c*; (– –) the NBS F^{III} component; (- - -) the NBS F^{II} component; (—·—) formyl-cytochrome *c*.

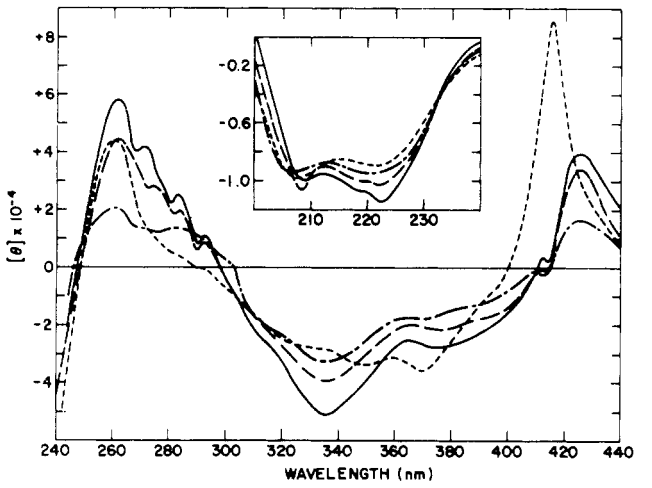

FIGURE 7: Circular dichroism spectra of native cytochrome *c* and modified preparations in the ferrous state of metal atom. Details same as in Figure 6.

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

Property	Preparation			
	Native	NBS F^{III} Component	NBS F^{II} Component	Formyl-Cytochrome
Spectra				
Ferric				
Soret (nm)	408	406	406	406
ϵ_{Soret} (mM)	104	113	122	94
ϵ_{695} (M)	166	28	NP[d]	NP
Ferrous				
Soret (nm)	416	418	417	417
ϵ_{Soret} (mM)	138	178	136	118
ϵ_{550} (mM)	27.4	23.8	19.2	ND
ϵ_{520} (mM)	15.9	14.5	13.2	ND
Reducibility				
Ascorbate	Fast	Fast	Slow	None
NADH–cytochrome *c* reductase (%)[a]	100	55	33	12
Oxidizability				
Oxygen	None	Slow	Fast	Fast
Cytochrome *c* oxidase (%)[b]	100	102	20	46
V_{max} with cytochrome *c* oxidase[c] (mM O_2/sec)	1.8	ND	ND	1.7
CO binding	None	Slow	Fast	Fast

[a] Determined according to procedure already described (Myer, 1972a). [b] Per cent of pseudo-first-order rate constant. Conditions: 1 ml of solution containing 3–10 μmol/l. of preparation; buffer 0.1 M phosphate (pH 7.0); reduced with solid borohydride; oxidation initiated with addition of 20 μl. of cytochrome *c* oxidase solution (3 μM heme *a*); reaction monitored at 550 nm. [c] Taken from Figure 1; Margoliash *et al.* (1973). [d] NP, not present; ND, not determined.

ranged heme coordination configuration. Like the NBS F^{II} component and formyl-cytochrome *c*, spectral type III of the NBS F^{III} component finally reverts to a native-like low-spin form at alkaline pH.

In the reduced state of the metal atom at neutral pH, all the modified preparations exhibit typical low-spin cytochrome *c* type hemochrome spectra, *i.e.*, a Soret band above 415 nm, a well-developed α band at 550 nm, and a β band at 520 nm (Table III). The spectroscopic characteristics of each modified preparation are not only distinct from those of the native protein, but also from one another. In each case the Soret is shifted to the blue by 1–2 nm with alteration of extinction, and the α and β bands consistently reflect lowered absorptivity. These spectral differences are indications that even in the reduced state of the metal atom, all of these modified preparations maintain a certain degree of perturbation of the heme group. Whether or not this involves variations in the coordination configuration of the metal atom could not be discerned from the results reported here. However, the spectroscopic properties, especially the position of the Soret band and its extinction, and the $\epsilon_{550}/\epsilon_{520}$ of both the NBS F^{II} component and formyl-cytochrome *c* in the ferrous state, are very similar to those of heme-peptide systems such as H8PT, H65, and H8PTn in the presence of imidazole, all of which are known to have a coordination configuration of His-18-Fe^{2+}-imidazole (Myer and Harbury, 1973; MacDonald, 1974; Y. P. Myer and H. A. Harbury, unpublished data). On the other hand, the spectral characteristics of the NBS F^{III} component do not conform to any of the characterized model systems nor to the other modified preparations. Since in the ferric state of the metal atom the NBS F^{III} component maintains a native-like coordination configuration between pH's 4 and 8 (see preceding section), and since it is well known that the affinity of ferrous heme iron for methionine-S coordination is two to three orders of magnitude higher than that for ferric forms (Harbury *et al.*, 1965), it is likely that the reduced NBS F^{III} component has a coordination configuration of the native protein, and the spectral differences are merely a reflection of small perturbations of the electronic configuration of the porphyrin moiety, possibly because of a slight loosening of the heme crevice.

Circular Dichroism (CD) Spectra. In comparison to absorption spectroscopy, circular dichroism measurements

provide a direct and ultra-sensitive probe for the conformational characterization of the molecule (Myer, 1968a,b). The alterations in the intrinsic absorption region reflect in general the variation in the polypeptide organization; the spectrum in the Soret region provides insights into the electronic configuration and conformation of the heme group; the δ-absorption region is associated with contributions from the porphyrin moiety; and the aromatic region permits evaluation of the microenvironment of the aromatic side chains (Myer, 1968a,b,1969). Figures 6 and 7 invite such a comparison of the three modified preparations with the native protein in both valence states of the metal atom.

Except for small decreases in the ellipticities of almost all the inflections, the CD spectrum over the entire range of the ferrous NBS F^{III} component is more or less indistinguishable from that of the native protein, suggesting the integrity of the native-like conformation. Although in the ferric state the preparation exhibits an unaltered intrinsic spectrum, there are definite alterations in both the visible and the near-uv regions (Figure 6). The lowered ellipticity at the 418-nm inflection with a concurrent increase at the positive 404-nm peak, the increased dichroic absorption in the region 300–360 nm, the unaltered 281- and 289-nm bands, the replacement of the doubly inflected spectrum in the region 250–260 nm by a single-peaked spectrum, and the small decrease at 222 nm with a slight blue shift and increased dichroic absorption at 208 nm are all characteristic changes associated with the first thermal denaturation step of the native protein (Myer, 1968a). These dichroic changes have been interpreted to be a reflection of the uncoupling of heme-polypeptide interactions without alteration of either the overall polypeptide conformation or of the coordination configuration of the molecule.

In contrast to the F^{III} component, the NBS-cytochrome *c* F^{II} component and formyl-cytochrome *c* in both valence states of the metal atom exhibit a dichroic spectrum with features typical of the conformationally deranged native protein (Myer, 1968a,b). In the ferric state positively peaked Soret spectrum, the absence of details in the δ-absorption region and the poor resolution of the dichroic peaks in the near-uv region (Figures 6 and 7) are typical of the ferric native protein in the presence of denaturing agents such as 9 M urea (Myer, 1968a) and of ferrocytochrome *c* in the presence of 4.5 M guanidine hydrochloride. Under these conditions both the ferric and ferrous forms of the native protein have been shown to undergo significant conformational changes, including the alteration of the nature of the centrally coordinated complex. The visible and near-uv CD spectra of formyl-cytochrome *c* in both valence states of heme iron are very similar to the CD spectra of simple heme-peptide models, such as H65, a heme-containing 65-amino-acid fragment from the native protein (MacDonald, 1974; Corradin and Harbury, 1971), and H11P, heme-undecapeptide from the parent molecule, as well as the simplest of the heme-peptide models, H8PT (heme octapeptide), in the presence of imidazole (MacDonald, 1974). Likewise, there are gross similarities among the CD spectra of the ferric and ferrous NBS F^{II} component and those of the model systems in the presence of extrinsic liganding molecules of low ligand-field strengths (MacDonald, 1974). Thus the NBS F^{II} component and formyl-cytochrome *c* in both valence states are molecular forms with a deranged polypeptide conformation, altered coordination configuration of the metal atom, and an appreciably loosened heme crevice.

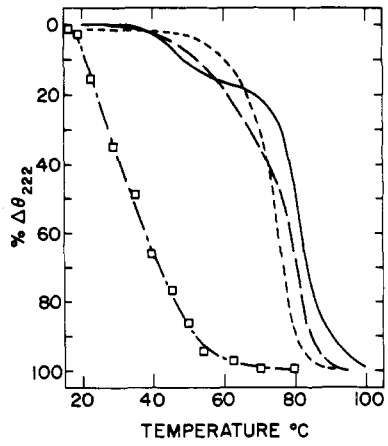

FIGURE 8: Thermal denaturation profiles of native cytochrome *c* and modified preparations in ferric state of metal atom; pH 7.0, in water. (—) Native cytochrome *c*; (- -) the NBS F^{III} component; (- - -) the NBS F^{II} component; (——-) formyl-cytochrome *c*. Measurements of formyl-cytochrome *c* at 404 nm.

In terms of the relative conformations of the NBS F^{II} component and formyl-cytochrome *c*, there are enough significant differences in their CD spectra to permit one to conclude that the two preparations are conformationally distinct in both valence states of the metal atom. In the ferric form, the NBS F^{II} component exhibits a Soret ellipticity about twice the magnitude of that observed in formyl-cytochrome *c*, the dichroic features in the near-uv and the uv regions are more or less obliterated in the latter (Figure 6), and differences in the CD spectra of the reduced forms are also evident in Figure 7. The conformational differences between the two preparations are apparently a reflection of the differences in the nature of the chemical modification, although in general terms both of these preparations exhibit a deranged coordination configuration.

Thermal Denaturation. The nature and the magnitude of the conformational derangement of a protein can also be inferred from the behavior of the system toward thermal denaturation. In the case of cytochrome *c*, thermal denaturation has been shown to differentiate between a localized conformational alteration in the vicinity of the heme group, *i.e.*, the first thermal denaturation step, and alterations in the polypeptide conformation, the second denaturation step (Myer, 1968a). In Figure 8 are compared the thermal denaturation profiles of the native protein, and two NBS-cytochrome *c* components and formyl-cytochrome *c* in the ferric state of the metal atom. The single symmetrical denaturation profile of the NBS F^{II} component, with the midpoint at about 74°, approximately 8° lower than the second step melting temperature of the native protein (Myer, 1968a), clearly describes a conformation with not only the absence of heme-polypeptide interaction, reflected by the absence of the first denaturation step (Myer, 1968a), but also, lowered stability of the protein conformation with respect to the native protein. In contrast, a lowering of about 47° in the melting temperature of formyl-cytochrome *c* definitely indicates the extent of the destabilization of the protein conformation resulting from modification of the single tryptophanyl residue. The persistence of pronounced asymmetry in the lower temperature regions, possibly a residual for the first denaturation step, and the lowering of the melting temperature by only 4° in the case of the NBS F^{III} cytochrome *c* component is in agreement with the inference that it is a molecular form with a practically unal-

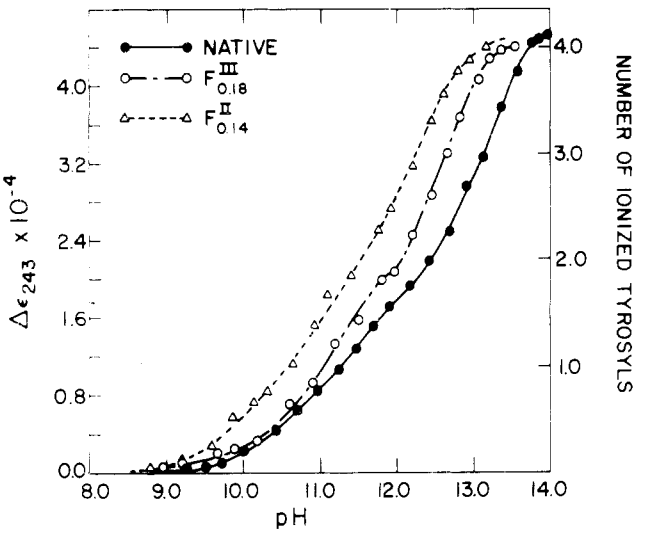

FIGURE 9: Spectrophotometric titrations of phenolic hydroxyls based on changes at 243 nm of native cytochrome *c* and the NBS F^{III} and F^{II} components. Conditions: solutions in 0.1 M NaCl; pH adjusted with NaOH.

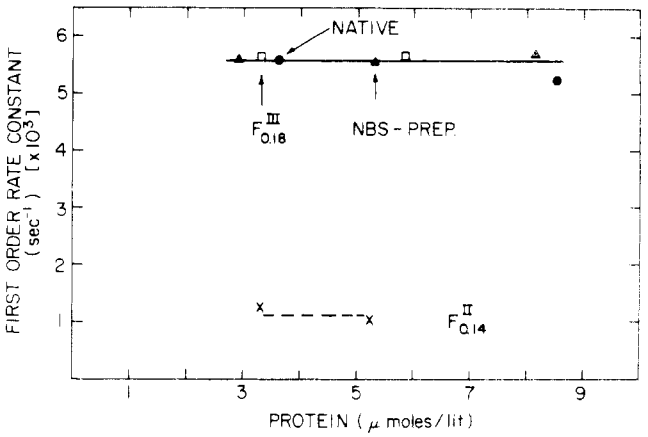

FIGURE 10: Plot of pseudo-first-order rate constant for oxidation of borohydride-reduced native cytochrome *c* and the NBS-unpurified preparation and its F^{III} and F^{II} components. Reaction conditions described in Table III.

tered polypeptide conformation with only a minor, although genuine, weakening of the heme–polypeptide interactions.

Spectroscopic Titration of Phenolic Hydroxyls. The proton-linked functions of phenolic hydroxyls of the protein and the modified preparations provide an additional probe for the conformational characterization of the system at the microscopic level, *i.e.*, in terms of the microenvironment of these functional groups. The variation of extinction at 243 nm during the alkaline titration of proteins has been shown to reflect primarily the alterations resulting from deprotonation of the phenolic hydroxyl groups (Stellwagen, 1964). The results from such measurements, both as changes in extinction and the proportion of tyrosyl side chains ionized at each level of titration for the native protein and the two NBS–cytochrome *c* components in the ferric state of the metal atom, are shown in Figure 9. All four tyrosyl side chains are titrated in the pH range 9–14 in both NBS components. The titration profile of the NBS F^{III} component is ascribed to the partial normalization of at least one of the three abnormal tyrosyl side chains, and the profile of the NBS F^{II} component reflects the further normalization of at least one of the tyrosyl side chains. The increasing normalization of the tyrosyl side chains from the NBS F^{III} component to the F^{II} component clearly reflects the increasing conformational perturbation of the molecule. Partial normalization of a single tyrosyl side chain in the NBS F^{III} component and the further normalization of tyrosyl groups upon modification of the Met-80 side chain in the NBS F^{II} component implies that although the two modified forms of the protein are conformationally perturbed, the NBS F^{III} component contains changes which are orders of magnitude smaller than those present in the NBS F^{II} component.

Enzymatic Activity and Other Chemical Properties. Like the native protein, both NBS–cytochromes *c* are reduced by ascorbate under anaerobic environment. The reduction of the NBS F^{II} component is orders of magnitude slower; *i.e.*, it requires as much as 24–36 hr for complete reduction, in comparison to the relatively short time for the native protein or the NBS F^{III} component (Table III). In contrast to the NBS–cytochromes *c*, formyl–cytochrome *c* was found to be nonreducible with ascorbate. All three preparations when stored in lyophilized form in the dark exhibited partial reduction, which is inherent to the native protein. In the reduced form all three preparations exhibit the tendency to form a complex with CO as well as to be autoxidized with molecular oxygen, properties which are absent in the native protein. Both the formation of the CO complex and the autoxidation of the NBS F^{III} component were exceedingly slow, whereas the NBS F^{II} component and formyl–cytochrome *c* underwent these reactions almost instantaneously. Since the affinity of the metal atom to form a complex with CO and the autoxidation property are associated with an increased accessibility to the buried heme moiety, *i.e.*, a loosening of the crevice surrounding the heme group, the order of magnitude difference in the affinities of the NBS F^{III} component and the other two preparations indicates that the NBS F^{III} component must be minimally perturbed, whereas the heme crevice must be almost completely loosened in the other two preparations.

In terms of the biological functioning of cytochrome *c*, the NBS F^{III} component retains about 55% of its capability to capture an electron from the NADH–cytochrome *c* reductase system while exhibiting no detectable variation in the electron donor properties to the terminal enzyme of the respiratory system, cytochrome oxidase (Table III; Figure 10). The NBS F^{II} component, on the other hand, exhibits only 33% reducibility and is more or less incapable of efficiently transferring electrons to cytochrome *c* oxidase. The formyl derivative exhibits 10–15% of the reducibility property, while its capability to transfer an electron to cytochrome *c* oxidase is very like that of the native protein (Table II). The unchanged V_{max} indicates that formyl cytochrome *c* is capable of transferring an electron to oxidase as efficiently as the native protein, but the lowering of the pseudo-first-order rate constant, V_{max}/K_m, indicates that the overall efficiency is reduced because of an enhanced K_m, which essentially reflects the dissociation constant of the complex between cytochrome *c* oxidase and the preparation. The lowered stability of the complex is presumably a reflection of conformational effects, while the unchanged V_{max} is a clear indication of a more or less native-like activity of the modified preparation. Thus in terms of the two aspects of the biological function of cytochrome *c*, the NBS F^{III} component and formyl–cytochrome *c* are forms with a deranged, but not completely destroyed, reducibility function and with unaltered oxidizability properties of the native protein, whereas the NBS F^{II} component is a molecular

species with derangement of both the oxidizability and reducibility properties of the molecule.

Discussion

The earlier interpretation of the reaction of NBS with ferricytochrome *c* lead to a suggestion that the modification of the protein occurs in a consecutive manner, first with the alteration of Met-65 and Trp-59, and finally resulting in a product containing the added modification of Met-80 (Myer, 1972a). The results reported here are confirmatory of the earlier interpretations of the course and nature of the NBS–cytochrome *c* reaction. This is evident from the observations that (i) the NBS–cytochromes *c* have been resolved into two major components (Figure 1); (ii) the relative proportions of the two components upon increasing modification of the protein exhibit a pattern typical of a consecutive reaction (Figure 2); and (iii) the chemical characterization indicates that Met-65 and Trp-59 are modified in the first product, the NBS F^{III} component, and Met-80 is the additional modification in the final product, the NBS F^{II} component (Table I; see Results). The structural characterization of purified formyl–cytochrome *c* also substantiates the finding that the apparent modification of the protein is of the Trp-59 residue. The spectroscopic characteristics in the uv region and the observation that at alkaline pH the formyl derivative reverts to the native protein are further indications that the modification is the 1-formylindole derivative (Aviram and Schejter, 1971; Previero *et al.*, 1967). The NBS treatment of the protein results in the oxidation of the methionines to the corresponding sulfoxides, and of tryptophan to the 5-bromo-2-oxindole derivative. The latter is inferred from the nature of the uv spectrum of the protein shown in Figure 3, which is similar to that observed for well-characterized tryptophan-containing model systems (Witkop, 1961; Green and Witkop, 1962).

As to the question of whether the modification of Met-65 or of Trp-59 or of both is responsible for the conformational and functional alterations, the detailed consideration reported earlier (Myer, 1972a) demonstrates clearly that the conformationally and functionally significant alteration in the NBS F^{III} component is that of Trp-59. The further alteration of properties exhibited by the NBS F^{II} component must be attributed to the modification of Met-80, since it is the only observable additional chemical change in this form of NBS–cytochrome *c*. The situation in formyl–cytochrome *c* is apparent, as it contains a single-site alteration of Trp-59 (Aviram and Schejter, 1971).

A comparison of the physicochemical properties of the NBS F^{III} and NBS F^{II} components with those suggested for the two NBS forms, form I and form II (Myer, 1972a,b; Myer and Pal, 1972), leaves little doubt as to the identity of the observed *vs.* suggested forms of the modified preparations. The NBS F^{III} component in the ferric state of the metal atom is a native-like molecule, except for a slight loosening of the heme crevice. The native-like behavior is reflected by the facts that the NBS F^{III} component exhibits a typical low-spin native spectrum, including the presence of the 695-nm band in the pH region 4–8 (Figures 4 and 5; Table II); there is only a slight normalization of one of the four tyrosyl residues (Figure 9); and it exhibits an intrinsic CD spectrum indistinguishable from that of the native protein (Figure 6). The presence of small and finite perturbations in the heme crevice is indicated by the following: the Soret CD spectrum is partially normalized (Figure 6), the Soret absorption maximum is shifted to the blue with enhancement of absorptivity (Figure 5), and the denaturation profile lacks resolution of the first denaturation step (Figure 8), which has been attributed primarily to the uncoupling of the heme–polypeptide interactions (Myer, 1968a). A similar consideration of the NBS F^{II} component indicates that it has a deranged coordination configuration of the metal atom and a small, finite alteration of the polypeptide conformation, all of which is consistent with expectations concerning the nature of preparations containing the modification of the centrally coordinated Met-80 side chain (MacDonald, 1974). All the physicochemical measurements reported here affirm this assessment of the nature of the conformational and configurational state of the NBS F^{II} component.

On one hand, the NBS F^{III} component in the reduced state exhibits absorption characteristics different from those of the native protein, the Soret band shifted to the red by 2 nm with enhanced absorptivity, and the α and β bands exhibiting a ratio of 1.6 instead of 1.7. It also has a slight tendency to form the CO complex and is slowly oxidizable with molecular oxygen (Table III). On the other hand, the dichroic measurements fail to provide any noticeable differences which could be construed to imply the alteration of the conformation of the molecule. An interpretation of the conformational state of the reduced NBS F^{III} component, consistent with the view of its ferric conformational state, is that it also represents a molecular form with a more-or-less unaltered protein conformation, but with small, finite perturbations in the electronic conformation of the heme group, possibly a slight loosening of the heme crevice. The CD spectrum of the reduced NBS F^{II} component (Figure 7) is typical of the deranged native protein, which in itself defines its conformational state.

The conformational situation of formyl–cytochrome *c* presents a picture distinct from either of the NBS–cytochrome *c* components. Although the preparation contains the modification of the Trp-59 residue only, which is the conformationally operational modification in the NBS F^{III} component, the pH–spectroscopic profile (Figure 4), the spectroscopic characteristics (Table II), and the intrinsic CD spectrum (Figure 6) are very similar to those observed for the ferric NBS F^{II} component. The dichroic spectra in the Soret and in the 240–340 nm regions, however, are not similar to that of the NBS F^{II} component (Figure 6) nor to any of the dichroic curves resulting from physicochemical perturbation of the native protein (Myer, 1968a). Thus, as to the conformational state of ferric formyl–cytochrome *c*, it is difficult, if not impossible, to draw any conclusions in terms of the specific nature and magnitude of perturbations. However, a number of facts permit one to state that ferric formyl–cytochrome *c* is a molecular form with an altered coordination configuration of the metal atom, as well as a grossly deranged molecular conformation. Among these are the observations that formyl–cytochrome *c* lacks the 695-nm band, characteristic of the Met-80–S–Fe linkage (Table II); the intrinsic CD spectrum reflects the occurrence of a small conformational denaturation of the polypeptide conformation (Figure 6); the Soret CD spectrum is simplified to a single symmetrical peak (Figure 6), typical of the native protein with a loosened heme crevice (Myer, 1968a); and the preparation exhibits a 47° lowering in its melting temperature (Figure 8). Considerations like those outlined above when applied to the physicochemical properties of the preparation in the ferrous state of the metal atom lead to a similar conclusion regarding its con-

formational state.

An explanation of the differences in the nature and the magnitude of conformational perturbations resulting from the modification of Trp-59 with NBS in the NBS F^{III} component, and those observed in the formylated preparation, apparently lies in the nature and position of the substituent groups. X-Ray data show that in both the ferric and ferrous states of the metal atom, Trp-59 is buried deep in the heme crevice and is involved in internal hydrogen bonding with the buried rear heme propionic acid side chain (Takano *et al.*, 1973). Formylation at position 1 of the indole moiety, the nature of the modification in formyl-cytochrome *c* (Aviram and Schejter, 1971), abolishes its ability to form the internal hydrogen bond, thus resulting in gross conformational perturbation of the molecule with the net effect, the disruption of the central coordination complex. *N*-Bromosuccinylation, on the other hand, yields the 5-bromo-2-oxindole derivative, which can be expected to result in the delocalization of the indole moiety, with possibly a slight weakening of the internal hydrogen bond of the indole nitrogen with the buried propionate side chain. The observation that the NBS F^{III} component shows only a slight perturbation of the heme environment, whereas formylation results in the complete disruption of the conformation of the molecule, especially in the immediate vicinity of the heme group, clearly establishes not only the importance of the Trp-59–propionate hydrogen bond in maintaining the heme crevice and the coordination configuration of the protein but also indicates that the hydrogen bond probably persists in the NBS F^{III} component, although the indole moiety of Trp-59 has been modified.

The ionization with a pK of 6.5 of the ferric NBS F^{III} component produces spectroscopic changes which are very similar to those associated with a transition in the native protein centered at a pH of about 9.1. The latter has recently been kinetically analyzed, and it has been suggested that it reflects two processes, a deprotonation step with a pK of 10.4 followed by conformational reorganization with an apparent pK of -1.3 (Czerlinski and Bracokova, 1971). The observation of a similar transition in the tetranitromethane derivative, with a pK of 5.9, which contains the modification of Tyr-67, while taking into account that a lowering of the pK by about 3 pH units is expected in the case of nitrated phenols, has lead Schejter *et al.* (1970) to suggest the involvement of one of the tyrosyl residues, possibly 67, in this ionization phenomenon in both the native and the nitrated preparation. The presence of the same phenomenon in the behavior of the NBS F^{III} component, which contains no modification of the tyrosyl side chains and which exhibits an almost native-like ionization of the four tyrosyl residues (Figure 9), makes it improbable that the proton-linked function is associated with one of the tyrosyl residues. Since the apparent perturbation in the NBS F^{III} component is a slight loosening of the heme crevice, the 2.7 pH unit shift in the pK for the disruption of the Met-80–S–Fe linkage may be a direct consequence of conformational perturbations rather than a shift in the pK of a specific functional group. In view of the significant role the Trp-59–hydrogen bond plays in the maintenance of the integrity of the heme conformation and because its disruption has been shown to result in the cleavage of the Met-80–S–Fe linkage, as in formyl-cytochrome *c*, it is possible that it could be the locus of the ionization under consideration.

In terms of the two aspects of cytochrome *c* activity, reducibility and oxidizability, the apparent effect of structural change in the NBS F^{III} component and formyl-cytochrome *c* is the selective alteration of only the reducibility function of the molecule. The added modification of Met-80 in the NBS F^{II} component results in not only a greater decline in reducibility, but also almost complete elimination of the oxidizability property (Table III). The selective derangement of reducibility in the NBS F^{III} component, and the alteration of both aspects in the NBS F^{II} component, are consistent with the behavior of the NBS preparations upon increasing modification of the molecule, as well as with the inference that the two aspects of cytochrome *c* function are indeed differentiable from one another (Myer, 1972a). The selectivity of the two aspects is not restricted to the NBS preparations only, as it is also the situation in formyl-cytochrome *c* (Table III), the purified tetranitromethane derivative with apparent modification of Tyr-67 (Pal *et al.*, 1974), modifications involving Tyr-74 (Morrison *et al.*, 1971; Margoliash *et al.*, 1973), and the carboxymethylated preparations containing substitution at Met-80 alone (Margoliash *et al.*, 1973) or in combination with Met-65 (MacDonald, 1974), as well as one containing modification of Lys-13 (Margoliash *et al.*, 1973). Recently, a similar observation has also been reported by Aviram and Schejter (1973) for the pyridoxal phosphate modified preparation of the protein. Since the selectivity of reducibility and oxidizability is manifested through chemical modification of rather different functional groups in the molecule, and since the X-ray diffraction data show that these functional groups not only lie in two different sections of the molecule, but also are located both inside and on the surface (Dickerson *et al.*, 1971; Takano *et al.*, 1973), an interpretation consistent with the foregoing involves the localization of the two aspects of cytochrome *c* activity in two distinct regions of the molecule which are appreciably separated from one another such that the functioning of one is more or less independent of the effective alteration of the other. This is essentially the two-path, two-function model for the functioning of cytochrome *c* as proposed earlier (Myer, 1972a), which has also received support from the finding that selective blocking of reducibility is also possible by a suitable antibody Fab fragment (Smith *et al.*, 1973), as well as from X-ray data (Takano *et al.*, 1973), which provide a molecular rationale for the irreversibility of the reduction pathway and for the reducibility function of the protein.

The maintenance of an unaltered oxidizability function in the NBS F^{III} component and formyl-cytochrome *c*, which differ from one another not only in the coordination configuration of the metal atom, but also in the conformation, indicates that as far as the oxidizability property of the protein is concerned, neither the integrity of the Met-80–S–Fe linkage nor the strict conformational form of the molecule are necessary. This is consistent with the earlier observation of carboxymethylated preparations, one containing the modification of Met-80–S (Margoliash *et al.*, 1973), and the other, of both methionyl side chains (MacDonald, 1974). These preparations have been shown to lack not only the Met-80–S–Fe linkage in both the ferric and the ferrous states (Keller *et al.*, 1972), but also to be conformationally deranged (MacDonald, 1974), but they do not exhibit a normal reaction with cytochrome *c* oxidase. Thus whether or not the reduced form has the Met-80–S–Fe linkage, or whether the oxidized form has a coordination configuration like that of the native protein, it seems to make little difference as far as the oxidizability function of the protein is concerned. The above considerations rule out the feasibility

of any model for the oxidoreduction of cytochrome *c* which involves the migration of an electron through the Met-80–S–Fe linkage (Dickerson *et al.*, 1972; Salemme *et al.*, 1973). The selectivity between the reducibility and oxidizability points to problems in acceptance of a reversible model as well. The lack of strict conformational requirements for the oxidizability function, however, implies that the donation of the electron could be through the exposed edge of the heme group, as has been suggested from studies of the oxidoreduction process with inorganic donors and acceptors (Yandell *et al.*, 1973; Creutz and Sutin, 1973).

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