Spectroscopic Properties of *N*-Bromosuccinimide-Modified Horse Heart Cytochrome *c*†

Yash P. Myer* and Pranab K. Pal

ABSTRACT: The *N*-bromosuccinimide-modified horse heart cytochromes *c* in the oxidized state of heme iron in the pH range 2–10 exhibit four different spectroscopic types. In the acid medium (type I) as well as in the alkaline medium (type IV), the spectral characteristics of the modified preparations are very similar to those exhibited by the native protein under identical conditions. At intermediate pH's (pH 4–5, type II; pH 6–8 type III), there are two distinct molecular forms, the relative proportions of which change with increasing modification of the protein. The proportion of the first form, which exhibits low-spin characteristics over the entire pH region 3–9 much like those of the native protein, reaches a maximum value in the preparation at a 3:1 NBS/protein ratio, while the proportion of form II, which exhibits a high-spin spectrum at pH's 4–5 (type II) and changes to a low-spin form lacking the 695-nm band with a p*K* of about 5.4, increases monotonically. A similar two-molecular-form situation is also present in the spectra of the modified preparations in the reduced state of iron. The extent and the nature of alteration of functional groups in various modified preparations, and their relative NADH-reductase and succinate activities, have been related to the coordinational configuration of the two forms. Form I, containing modified tryptophanyl residue 59 and methionyl residue 65, exhibits a spectral behavior and succinate oxidase activity very similar to that of the native protein, but the NADH-reductase activity is deranged; form II is the molecular species produced in a consecutive manner by additional modification of methionyl residue 80; the coordination configuration of the heme iron is altered, which results in the loss of the succinate oxidase activity of the protein as well.

The elucidation of structure-biological function relationships in proteins through studies of effects of chemical modification of amino acid functional groups, of necessity, requires investigation of multiple facets: first, the structural localization of the chemical modification and studies of its effects on the biological function of the molecule; second, the structural interpretation of the reactivity of the various functional groups; and third, the implications of the chemical modification upon the conformation of the molecule. In the case of the reaction of horse heart ferricytochrome *c* with *N*-bromosuccinimide (NBS),[1] in the preceding two papers we have dealt primarily with these aspects of the investigations. However, in the system under question, cytochrome *c*, one important aspect—the effect of the chemical modification on the nature of the central-coordinated complex of the heme iron—needs further consideration, since it constitutes the seat of biological function, the electron-capture and the electron-donor properties in the electron-transport chain. In this article, we have been concerned with this facet of the structure–function relationships in this molecule.

Materials and Methods

Native horse heart cytochrome *c*, type III, was purchased from Sigma Chemical Co.; the NBS derivatives used for these investigations are those whose preparation and structural, functional, and conformational characteristics are reported in the preceding two articles (Myer, 1972a,b). The results from amino acid analysis and the relative NADH cytochrome *c* reductase and succinate oxidase activities are listed in Table I for each preparation used in these investigations.

Spectroscopic measurements were conducted on a Cary 15 spectrophotometer, and the pH measurements were made using a type B glass electrode and a Radiometer Model 26 pH meter. The titrations were conducted in a specially designed apparatus, the details of which are reported in a recent article (Myer and Harbury, 1972). The solutions during these investigations were maintained constantly under an inert atmosphere of ultrapure helium, and the addition of the reagent, acid or base, was made by ultraprecision syringes. The reduction of the solutions was performed by direct addition of solid dithionite to the apparatus, while the pH of the solution was continuously monitored and maintained automatically at a prescribed value with the aid of a Radiometer autotitrator. The concentrations of the solutions were determined spectrophotometrically using the appropriate extinctions at 506 nm, the isodichroic point for the NBS reaction (Myer, 1972a).

Results

The 3:1 NBS-modified ferricytochrome *c* between pH 2.0 and 10.0 exhibits four spectroscopically distinguishable types (Figure 1). Type I, a stable molecular form below pH 2, converts to type II with an apparent p*K* of about 3.0, which subsequently changes to type III with a p*K* of about 5.4. The stable type at neutral pH finally changes to type IV with a mid-pH of about 9.5. The four types exhibit stable pH regions of below pH 2.0, 4–5, 6–8, and at alkaline pH's, respectively. The visible spectra of the 3:1 NBS-modified preparation in the ferric state of heme iron corresponding to the four types are shown in Figure 2, and in Figures 3 and 4 the absorption curves for both the ferric and ferrous forms of each preparation are compared. The positions of the spectral bands are listed in

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

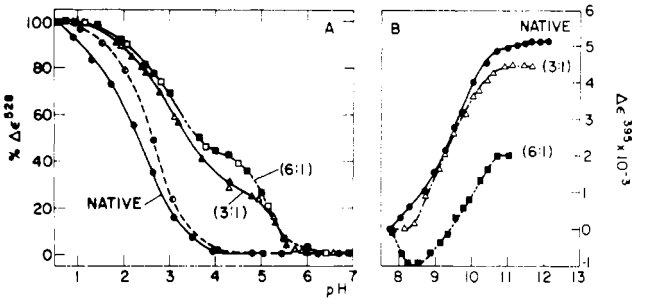

FIGURE 1: Spectroscopic titration of native and NBS–cytochromes *c* in acidic and basic pH ranges. (A) Acidic pH range; (B) basic pH range. In a part A, filled markings designate the titration from pH 7 to 1, and unfilled markings, the reverse titration. (○,●) Native; (△, ▲) 3:1 NBS-modified preparation; (□, ■) 6:1 NBS-modified preparation.

TABLE I: Amino Acid Content and Relative Activities of Various NBS Cytochromes *c*.

Prepn (NBS/Protein, Moles/Mole):	0.00 (Native)	3.01	6.02	9.00
Amino Acid Content (Moles/Mole of Protein)				
Tryptophan	1.0	0.02	0.00	0.00
Methionine sulfoxide[a]	0.0	1.3	1.6	1.8
Methionine[b]	2.0	0.7	0.4	0.2
Tyrosine[c]	4.0	3.3	2.7	2.1
Histidine	3.0	3.0	2.9	2.9
	Activity			
NADH–cytochrome *c* reductase activity (%ΔOD$_{550}$/min per μmole)[d]	100	28	32	26
Succinate oxidase activity (% rate at 6 μM concentration)[d]	100	68	30	<15

[a] Determined indirectly as methionine sulfone after carboxymethylation at pH 3.0 and performic acid oxidation. Zero was taken for the native protein when estimated by the above procedure and 100% was taken for the native protein when the carboxymethylation step was omitted. [b] Difference between the methionine sulfoxide content and the amount present in the native protein. [c] NBS data corrected for loss during hydrolysis. [d] For details, see Myer (1972a).

Table II for the ferric 3:1 NBS-modified preparation along with those of the native protein for ease of comparison.

Type I, observable in extremely acidic conditions, exhibits characteristics similar to those of the native protein under similar conditions. The position of the Soret maximum at 394 nm, a visible maximum at 495 nm with a well-defined shoulder at 530 nm, and the well-resolved 618-nm peak are all spectral features typical of the high-spin form of the hemoprotein (Day *et al.*, 1967) and simple heme–peptide systems (Myer and Harbury, 1972). Type II does not appear to have an analog in the spectroscopic behavior of the native protein, and it exhibits rather peculiar spectroscopic characteristics (Table II and Figures 2 and 3). It contains a peak at 528 nm with a well-defined shoulder at about 565 nm, and a visible minimum at 494 nm, features similar to those of the native protein (Table II), which is characteristic of the low-spin state of heme iron, on the one hand, and on the other, it has a well-resolved peak at about 615 nm (Figure 2), which is characteristic of the high-spin form of the protein. In addition, Type II exhibits a well-defined peak in the 690- to 700-nm region, a spectral feature attributed to the presence of methionyl sulfur coordination to heme iron (Schechter and Saludjian, 1967) and also to the low-spin state of the metal atom (Zerner *et al.*, 1966). The position of the Soret band, 403 nm as compared to 409 nm for the native protein at this pH, is also indicative of the complexity of the spectrum.

The transformation of type II to type III with a p$K \simeq$ 5.4 results in changes which eliminate the spectral pecularities of type II. The low-spin characteristics become more pronounced—*i.e.*, the 528-nm peak is enhanced and the Soret band is moved to 407 nm—with concurrent elimination of the high-spin band at 615 nm (Figures 2 and 3; Table II). The spectrum in the region 690–700 nm is more or less unaltered, except for a small decrease of extinction over the entire region (Figure 2).

Type IV retains essentially all the features of type III, except that the Soret band is shifted to the blue with enhanced absorptivity, and there is a slight lowering of extinction in the visible bands; type IV is devoid of any known characteristics of the high-spin state of heme iron. The most significant change is the elimination of the peak in the 690- to 700-nm region. The spectral changes associated with the transformation of types III–IV are similar to those observed for the native protein undergoing transition in the same pH range (Table II), and the spectral characteristics are typical of the low-spin form of cytochrome *c* lacking the criteria for the presence of methionyl sulfur coordination to heme iron, namely, the band in the region 690–700 nm (Schechter and Saludjian, 1967).

The spectrophotometric titration of the preparation at higher than 3 equiv of NBS exhibits approximately the same inflections in the pH–absorption curves as reported for the 3:1 NBS-modified preparation (Figure 1), with the only apparent difference being the alteration of the magnitude of ΔOD for various inflections. Spectroscopically both types I and IV are very similar to the spectrum of the 3:1 NBS-modified preparation as reported in Figures 2 and 3. Types II and III, however, exhibit variations in the spectrum, which, in the case of type II, are characterized by increasing absorptivity at the 618-nm band with concurrent decreases in the Soret and the 528-nm regions (Figure 3), in the order of increasing NBS/protein ratios. The same situation is seen in the spectral properties of type III. In the region of the 695-nm band, the resolution of the band becomes weaker with increasing NBS modification of the protein to such an extent that for preparations at NBS ratios of higher than 6 equiv, the band is barely discernable. From a comparison of the spectral characteristics of the native protein to those exhibited by the 3:1 NBS-modified preparation, on the one hand, and the nature of the differences among the 3:1, 6:1, and the 9:1 preparations, on the other (Figures 2 and 3), it seems that during the course of the NBS reaction, at least two spectroscopically distinguishable molecular forms are produced, possibly in a sequential manner, one forming mainly between 1 and 3 equiv of NBS, and the second, with reaction at higher reagent equivalents.

Types II, III, and IV, when reduced with dithionite under anaerobic conditions, exhibit spectra which are characteristic of the low-spin form of the reduced-iron configuration, as in

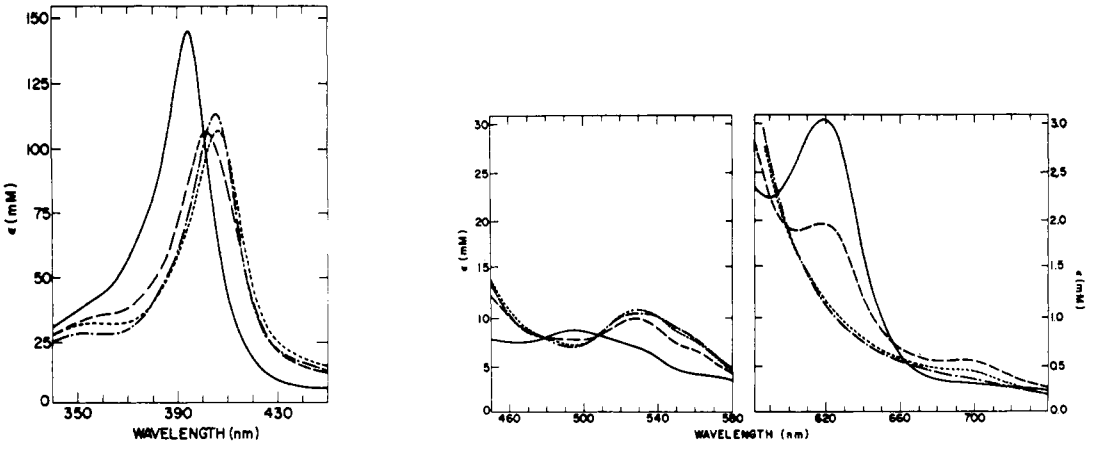

FIGURE 2: Absorption spectra corresponding to the four stable spectral types of 3:1 ferric NBS–cytochrome *c* preparation in the Soret (left) and visible (right) spectral regions. ———, type I, pH 2.0; – – –, type II, pH 4–5; - - - - -, type III, pH 6–8; –·–·–, type IV, pH 10.0.

the case of the native protein (Figure 4), but there are small and significant differences, both in the Soret and the visible regions of the spectrum. The spectral difference in the Soret region between the 3:1 NBS–cytochrome *c* and the native protein is merely the red shift of the Soret band with enhancement of absorptivity, whereas the difference between the 3:1 NBS–cytochrome *c* and other preparations is the lowering of absorptivity with apparently no noticeable change in the band position. In the visible region, on the other hand, NBS modification seems to result in a continuous decrease of absorptivity at both the α and β bands. This may be taken as a measure of either decreasing reducibility of the molecule or of increasing formation of irreducible forms or both. Since the final NBS-modified preparation, *i.e.*, 9:1, exhibits the characteristic α and β bands in the visible region, and since the ratio of extinction of the two bands is significantly different from that in the case of the native protein (1.4 as opposed to 1.8), the decreasing absorptivity in the visible region could result from an increasing proportion of the form characterized by 9:1 NBS–cytochrome *c*. Likewise, the spectral differences between the 3:1, 6.1, and 9:1 NBS-modified preparations in the Soret region could be reflections of increasing proportions of the final form produced by the NBS reaction.

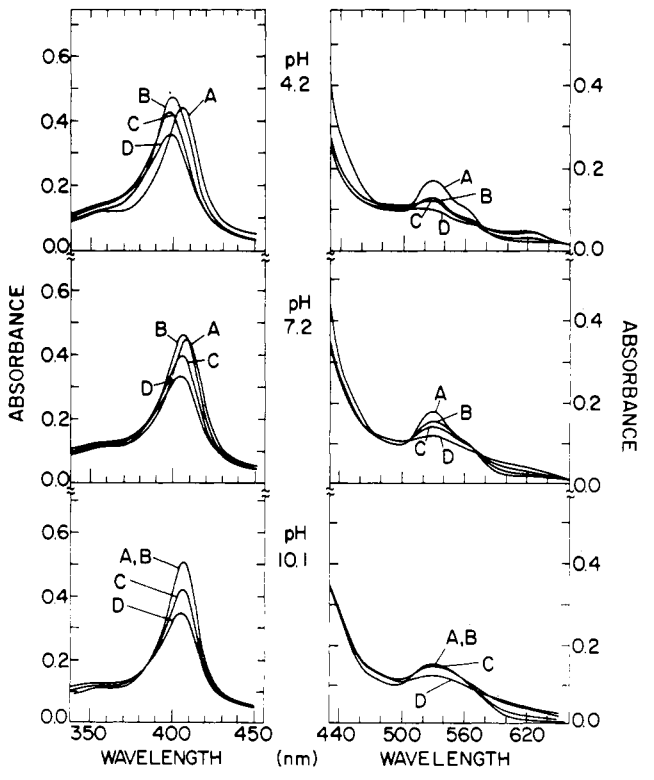

FIGURE 3: Absorption spectra of ferric native and NBS–cytochromes *c* at pH's corresponding to types II, III, and IV. pH 4.2, type II; pH 7.2, type III; and pH 10.1, type IV. (A) Native; (B) 3:1 NBS–cytochrome *c*; (C) 6:1 NBS–cytochrome *c*; and (D) 9:1 NBS–cytochrome *c*.

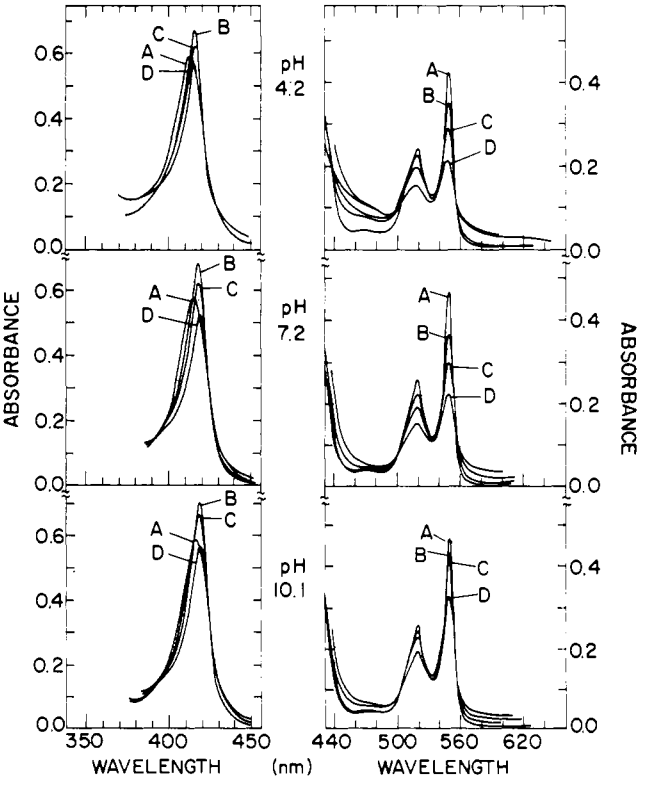

FIGURE 4: Absorption spectra of ferrous native and NBS–cytochromes *c* at pH's corresponding to types II, III, and IV. pH 4.2, type II; pH 7.2, type III, and pH 10.1, type IV. (A) Native; (B) 3:1 NBS–cytochrome *c*; (C) 6:1 NBS–cytochrome *c*; and (D) 9:1 NBS–cytochrome *c*.

TABLE II: Spectral Band Positions for Ferric Cytochrome *c* and Ferric 3:1 NBS-Modified Preparation at Different pH's Corresponding to Various Types.

pH:	≤2.0	4.5	7.0	≥10.0
Type:	I	II	III	IV
		Native Cytochrome *c*		
Absorption maxima (Sh)	618, 570, (530), 495, 394	695, (565), 528, 409, 365	695, (565), 528, 408, 365	(560), 528, 406, 365
		3:1 NBS–Ferricytochrome *c*		
Absorption maxima (Sh)	618, 570, (530), 495, 394	690, 615, (565), 528, 403, (365)	690, (565), 528, 407, 365	(565), 528, 406, 365

The spectral difference in the Soret region between the native and the 3:1 NBS-modified preparations, however, falls into a distinct category of its own, which could be an indication of the formation of an intermediate species with a Soret band at about 418 nm with enhanced absorptivity, finally changing to the form exhibited by the 9:1 preparation. Thus it seems that in the reduced state of heme iron as well, the spectral behavior of the NBS-modified preparation suggests the consecutive formation of at least two distinct molecular forms, the 3:1 NBS-modified preparation, reflecting in the main the characteristics of the intermediate molecular species, and the 9:1 NBS-modified preparation, the final molecular form of the modified protein.

Upon reduction of type IV, the resulting spectra for the 3:1 and 6:1 NBS-modified preparations in the visible absorption region are essentially similar to the spectrum of native ferrocytochrome *c* (Figure 4), whereas in the Soret absorption region, the positional difference is maintained. Since the α and β bands (the Q band) reflect mainly the contribution from the ligand–metal transition and are sensitive to the nature of the coordinating groups, while the Soret band (B band) is primarily a porphyrin π–π^* transition and sensitive to the environment of the porphyrin rings, and since the NBS reaction causes structural alteration of the functional groups which are in the immediate vicinity of the porphyrin moiety (Myer, 1972a; Dickerson *et al.*, 1971), the generation of identical visible spectra could mean that the coordination configuration of heme iron in type IV is similar, if not identical, to that of the native protein, and the Soret differences are simply reflections of effects associated with the structural changes of the protein moiety in the immediate vicinity of the porphyrin moiety. The small spectral deviation for the 9:1 NBS-modified preparation could be ascribed to additional structural alteration of the molecule in the region of 6–9 equiv of NBS, the details of which are discussed in the preceding article (Myer, 1972a).

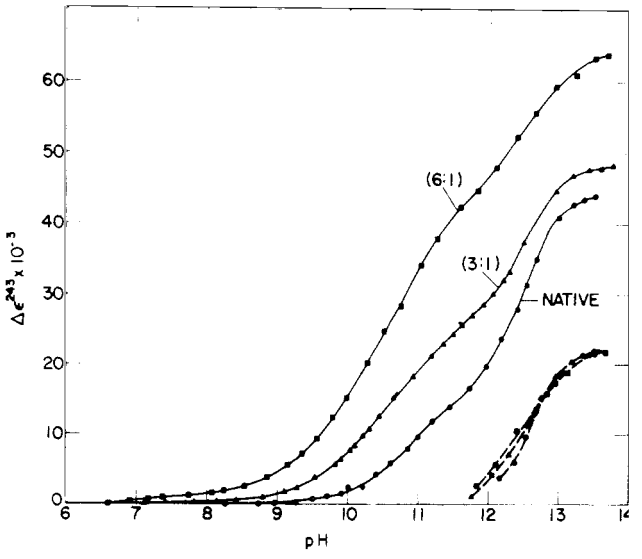

FIGURE 5: Spectrophotometric titration of phenolic hydroxyls of native and NBS-modified preparations. (●) Native; (▲) 3:1 NBS–cytochrome *c*; (■) 6:1 NBS–cytochrome *c*. Right bottom: normalized curves for the titrations of abnormal hydroxyls in native and modified preparations.

Spectophotometric Titration of Phenolic Hydroxyls. The variation of extinction at 243 nm during the alkaline titration of proteins has been shown to be a reflection of the spectral alterations resulting from the ionization of the phenolic hydroxyl of the tyrosyl side chains (Stellwagen, 1964); the results from such measurements for the native protein and the 3:1 and 6:1 NBS-modified preparations are compared in Figure 5. The results for the native protein are similar to those reported by Stellwagen and Van Rooyan (1967) and Rupley (1964), which have been resolved into a series of ionizations with apparent pK values of 10.2, 11.4, 12.6, and 12.6, respectively. The titration curve for the 3:1 NBS-oxidized preparation can also be fitted to almost identical apparent pK values, 10.2, 11.4, 12.6, and 12.6, with an enhanced $\Delta\epsilon_{243}$ for the first of the ionization groups. The titration curve of the 6:1 NBS-modified preparation, on the other hand, can be satisfied with two sets of identical pK values, *i.e.*, 10.2 and 12.6, but with enhanced extinction of tyrosyl residues with apparent pK's of 10.2.

The analysis of the phenolic titration curves in general indicates that the apparent pK values of two of the four residues, the residues ionizing with apparent pK's of 12.6, are unaffected by the modification of the protein. An additional tyrosyl group, the group with an apparent pK of about 11.4, is also unaffected in the 3:1 NBS-modified preparation. The former is further confirmed by the observation that the normalization of the three titration curves to yield a maximum $\Delta\epsilon_{243}$ of 22×10^3 M^{-1} $l.^{1}$ cm^{-1}, the expected variation for the ionization of two unmodified tyrosyl hydroxyls (Stellwagen, 1964), at the highest pH value, results in titration curves which are quite comparable to one another (see curves at the bottom right of Figure 5). Since the apparent pK's, especially of the abnormal phenolic hydroxyl group, provide a direct measure of the microconformational environment of the side chains of each residue, the lack of alteration of the pK of the abnormal

phenolic hydroxyls indicates that the conformational environment in the close vicinity of these groups is insensitive to structural (Myer, 1972a) as well as conformational changes (Myer, 1972b) in other parts of the molecule. Thus the conformational changes resulting from NBS modification, especially up to 3 equiv of reagent, must be localized in limited sections of the molecule, possibly in the close vicinity of the heme group, which has been independently inferred in studies reported in the preceding article (Myer, 1972b).

The observation that the 3:1 NBS-modified preparation exhibits enhanced extinction of the phenolic hydroxyl with an apparent pK of 10.2 could be taken as an indication of the molecular location of the residue undergoing modification during the early stages of the NBS reaction. The value of 10.2 is typical of a normal tyrosyl residue; the functional group under question must be the one exposed to the solvent. When examined with the information available from X-ray diffraction results (Dickerson *et al.*, 1971) and the results presented in the preceding article (Myer, 1972a), the tyrosyl residue susceptible to NBS modification during the early part of the reaction seems to be residue 74. The position of the second, with a pK of 11.4 and undergoing modification in the latter part of the reaction, could not be ascertained with any confidence from these studies. Further investigations are in progress to clarify this aspect of the results.

Discussion

The nature of the groups coordinating centrally to heme iron in cytochromes *c* is well established—*i.e.*, the two available positions of the central iron are occupied by the imidazole moiety of histidyl residue 18 and methionyl sulfur of residue 80 (Dickerson *et al.*, 1971; Fanger *et al.*, 1967; Harbury, 1966; Schechter and Saludjian, 1967; Harbury *et al.*, 1965). Spectroscopically the protein at pH's higher than 3 is low spin in form and is typified by the presence of a unique band at about 695 nm between pH 2.5 and 9.0 (Theorell and Åkesson, 1941). This band has been related to the binding of methionyl sulfur to heme iron (Schechter and Saludjian, 1967). Recently it was also ascertained that the intensity of the 695-nm band could be substantially lowered by conformational changes which involve exposure of the tryptophanyl and tyrosyl residues (Sreenathan and Taylor, 1971). Native ferricytochrome *c* reverts to another spectroscopic form at alkaline pH's (p$K \simeq 9.0$), which maintains all the typical low-spin spectral characteristics (528-nm band, Soret band at 407 nm, etc.), but the spectral criterion reflecting the sulfur–iron coordination, the 695-nm band, is absent. Below pH 3 the low-spin spectrum reverts to a high-spin spectrum, which in addition to the typical spectral characteristics (Soret band at 390–400 nm, and the visible maximum at 495 nm with a shoulder at about 565 nm), contains the 620-nm high-spin band (George *et al.*, 1961; Day *et al.*, 1967). The 620-nm band has recently been noted also in the spectra of model systems with a possible mixed-spin coordination configuration, *i.e.*, with one high-strength and one low-strength ligand-field group (Myer and Harbury, 1972). In the reduced state of heme iron, over the entire pH range, the protein exhibits a typical hemochrome spectrum, the Soret band at 416 nm, the α band at 550 nm, and the β band at 520 nm. These spectroscopic-structural relationships constitute the discussion of this article.

The typical high-spin spectra of all the NBS-modified preparations at acid pH's and their indistinguishability from those observed for the native molecule under identical conditions, in which both the modified preparations and the native protein are devoid of any significant secondary and ternary structures (Myer, 1968, 1972b), indicate that the structural integrity of the heme moiety is maintained during the course of NBS modification of the protein. The presence of an almost comparable situation at the other end of the pH scale, especially for preparations up to about 6 equiv of NBS (Figure 3), further supports the above view. The spectral and functional differences between the various modified forms and the native protein in the intermediate pH's therefore must be reflections of effects associated directly or indirectly with the NBS-inflicted structural alteration of the protein moiety.

The spectroscopic behavior of NBS preparations at 3 or more equiv of reagent in the oxidized state of heme iron in the pH region 4–8 can be adequately explained on the basis of the successive formation of two forms of NBS-modified cytochrome *c*. Form I contains modified tryptophan-59, methionine-65, and possibly one of the tyrosyl residues, and form II is the molecular species containing additional modification of methionine-80 (Myer, 1972a). Form I is low-spin throughout the pH region 4–8, with coordination configuration like the native protein. Form II, on the other hand, is high spin at pH 4–5, and changes with a pK' of 5.4 to a low-spin form which is configurationally distinct from either the native protein or form I. These conclusions have been reached on the basis of the following considerations.

The presence of at least two molecular forms in NBS preparations is indicated from the existence of two distinct types of differences in the Soret spectrum of oxidized NBS-modified preparations (Figure 3), and from the observation that increased modification of the protein results in a concomitant increase and decrease of the $\Delta\epsilon$'s corresponding to the two transitions with apparent pK's of 5.4 and 3 (Figure 1), neither of which has its counterpart in the pH transition of the native protein. Additional support to the above assertion for the most part comes from a consideration of the spectral changes of the type II complex with increasing modification of the protein. The type II spectra of the 3:1 and 6:1 preparations contain features which are characteristic of both the high- and the low-spin configurations of the heme iron, *i.e.*, the 620- and the 695-nm bands. With increased modification of the protein, the high-spin characteristics are enhanced, with a concurrent weakening and finally elimination of the features characteristic of the low-spin form of the molecule (Figure 3), a clear reflection of the transformation of the low-spin form to the high-spin form with increasing NBS modification. The 3:1 preparation contains a minimal proportion of the high-spin form, and a comparison of its low-spin characteristics, such as the position of the Soret band and the extinction of the 695-nm band, suggests that the low-spin form in type II cannot be due to the presence of unmodified native protein. The extinction at 695 nm, if taken to represent the proportion of the native protein in 3:1 NBS-modified cytochromes, suggests that in order to generate the observed spectral characteristics, as much as 50% of the modified molecule must be the native protein. In addition, since the high-spin characteristics predominate with a concurrent decrease in the low-spin features (Figure 3) with increasing modification, the inference is unavoidable that the change represents the formation of the modified molecular form at the cost of the native protein, if the stipulated sitation is the case. The consideration of spectral differences in NBS preparations in the Soret region, on the other hand, leads to an opposing solution (see preceding section). Because at the 3:1 equivalent stage of the NBS reaction there has been complete modification of a single tryptophan

residue and of methionine-65, and because the preparation exhibits a minimal NADH-reductase activity (Myer, 1972a), the speculation that the 3:1 NBS-modified preparation contains a significant fraction of unmodified protein is highly unlikely. The observed spectral properties of the 3:1 NBS preparation therefore can be adequately justified only if the low-spin characteristics are due to the formation of the first of the modified forms, and the increasing high-spin characteristics represent the successive formation of form II. Additional support to the above inferences also comes from a consideration of the spectral alterations associated with the transition with a pK' of 5.4 (Figure 1). These effects—*i.e.*, the transformation of a mixed-spin spectrum to a typical low-spin spectrum (Figure 2)—can be interpreted in two ways: (i) mixed-spin type II represents a state of equilibrium between two spin isomers of one molecular form, *i.e.*, a one-component, two-state system, and the pH change merely results in the shift of the equilibrium to one of the two isomers; or (ii) the NBS-modified preparations are indeed two-component systems, forms I and II, and the pK is a reflection of the transformation of high-spin form II in type II to a new low-spin form in type III. The former explanation has been proposed for the mixed-spin spectral behavior of ferric myoglobin hydroxide (George *et al.*, 1961). In the present situation, this possibility can be ruled out, not only from the evidence presented in the previous paragraphs, but also by the fact that the spectral alterations associated with a pK of 5.4 do not conform to the changes expected for a one-component, two-isomer system. Two significant reasons are: (i) no isosbestic points exist among the spectra of type I, the high-spin limit, type II, the intermediate form, and type III, the final state of the equilibrium process (Figure 2); and (ii) although the transformation of mixed-spin type II to low-spin type III results in the elimination of the high-spin form, reflected by the elimination of the 620-nm band (Figure 2), there is no enhancement, actually a slight lowering, of the absorptivity of the 695-nm band, which characterizes the coordination configuration of the low-spin form in type II. This is contrary to expectations of a two-isomer, one-component equilibrium change. Therefore, of the two possibilities, the latter is evidently the appropriate explanation of the spectral properties of the NBS-modified cytochromes. The second rationale also implies that, since there is no further contribution to the 695-nm band, which is specific in terms of the coordination structure of the heme iron (Schechter and Saludjian, 1967), the resulting low-spin moiety with a pK of 5.4 (low spin of form II) must be of a different coordination configuration, *i.e.*, it must be lacking the Fe–S linkage which generates the 695-nm band. Also the very fact that the 695-nm band persists over this pH transition indicates that low-spin form I in type II is insensitive to the pH changes in this range of the scale; furthermore it must have a coordination configuration very similar to that of the native protein, the Fe–S linkage. This behavior is indeed similar to that of the native protein. Support to the above-assigned coordination configuration also comes from the structural characterization of the products (Myer, 1972a), *i.e.*, form I contains modification of both methionine-65 and tryptophan-59, but methionine-80, which provides the sixth coordinating group in the native protein (Schechter and Saludjian, 1967; Dickerson *et al.*, 1971), is unaltered, and form II represents the form with added modification of methionine-80. Conformational studies provide independent support to the foregoing conclusion (Myer, 1972b) by indicating that only in the latter case is the nature of the centrally coordinated complex chemically altered.

In the case of ferrous NBS-modified cytochromes, over the entire pH range 4–10, the initial red shift of the Soret peak with 20–30% enhanced absorptivity of the 3:1 NBS-modified preparation, and the subsequent continuous lowering of extinction with little or no change in the position of the band with increased modification of the protein (Figure 4), represent a situation identical to that of the spectral differences in the case of the ferric NBS cytochromes (Figure 3). Thus, the ferrous NBS-modified preparations at 3 or more equiv of NBS are composites of two spectroscopically distinct molecular forms of the modified protein. Since all the preparations exhibit typical low-spin hemochrome spectra over the entire pH region (*i.e.*, the Soret band at about 418 nm with well resolved α and β bands), the low-spin coordination configuration for both forms is evident.

In contrast to the nature of spectral differences in the Soret region between the native and the ferrous NBS preparations (Figure 4), the spectral differences in the region of the α- and β-absorption bands reflect a continuous change with increasing modification of the protein, which, if taken at face value, could lead to conclusions contradictory to those in the earlier part of the discussion, *i.e.*, the continuous lowering of the extinction with increasing modification of the protein could be taken to reflect that NBS reaction results in the formation of a single molecular form. The apparent contradiction can be resolved if the following interpretation is accepted—that the decreased extinction in the visible region of the reduced NBS-modified preparations (Figure 4) is due to increasing concentration of form II, which is typified spectroscopically by the spectrum of the 9:1 preparation. A corollary of the preceding is that form I in this region of the pH scale and in this region of the spectrum is spectroscopically much like the native protein. In view of the earlier conclusion that form II in the ferric state of the heme iron is typified by the magnitude of the 620-nm band in the type II complexes, the validity of the above interpretation can be established by comparison of the estimated proportions of form II, based on the magnitude of the lowering of extinction of the α band in the ferrous state of the NBS preparations, with similar estimates in the ferric state of the heme iron based on the high-spin characteristics of form II, the 620-nm band in the type II complex. As shown in Table III, estimates of form II, whether from spectral changes in the α band of both type II and III complexes, or from the magnitude of the 620-nm band in the type II complex in the ferric state of the molecule, yield identical results. Thus the NBS cytochromes in the reduced state of the heme iron are also mixtures of two distinct modified forms over the pH region 4–10. The results in Table III also indicate that form II in the ferric NBS-modified preparation, which is of high-spin form at pH 4–5 and changes to a low-spin form with a pK of 5.4, exhibits an altered reduced spectrum in the visible region; form I, the low-spin form containing coordinated sulfur of methionine-80, is spectroscopically indistinguishable from the native protein in the visible region of the spectrum in the ferrous state of the heme iron. The altered Soret spectrum of this form is consistent with the conclusion that, although this form maintains the same coordination configuration as the native protein, distinct changes in the electronic environment of the prosthetic group have occurred (Myer, 1972b).

Based on the above conclusions, the interpretation of the spectroscopic behavior of the two forms of NBS-modified cytochromes, in terms of their coordination configurations of the heme iron, is schematically represented in Figure 6. Both forms at very low pH's exist in the high-spin configura-

TABLE III: Methionyl Content of NBS Preparations and Spectroscopically Estimated Proportions of the Two Molecular Forms in Both Valence States of Heme Iron.

Prepn NBS/ Protein (moles/ mole)	Fraction of High-Spin Form in Type II[a]	Fraction of Mol Form Resulting in Lowering of Extinction at 550 nm in Types		Methionyl Content
		II[b]	III[b]	
3.01	0.32 (0.68)[c]	0.37 (0.63)	0.42 (0.58)	0.7
6.02	0.64 (0.36)	0.65 (0.35)	0.66 (0.34)	0.4
9.00	0.94 (0.06)	1.00 (0.00)	1.00 (0.00)	0.1

[a] Based on optical density (OD) at the 620-nm band for equivalent concentration of native and NBS-modified preparations at pH 4.5. The expression used was: fraction of high-spin form = $((OD_{620}^{obsd} - (OD_{620}^{native})/((OD_{620}^{native}$ at pH 2.0 $- (OD)_{620}^{native})$. [b] Based on optical density at the 550-nm band for equivalent concentrations of native and modified preparations in the reduced state of the preparations. Expression used: fraction of form resulting in decrease of absorptivity $= ((OD)_{550}^{native} - (OD)_{550}^{obsd})/(OD_{550}^{native} - (OD)_{550}^{9:1\ prepn})$. [c] Values in parentheses are the difference from unity: the proportion of form I.

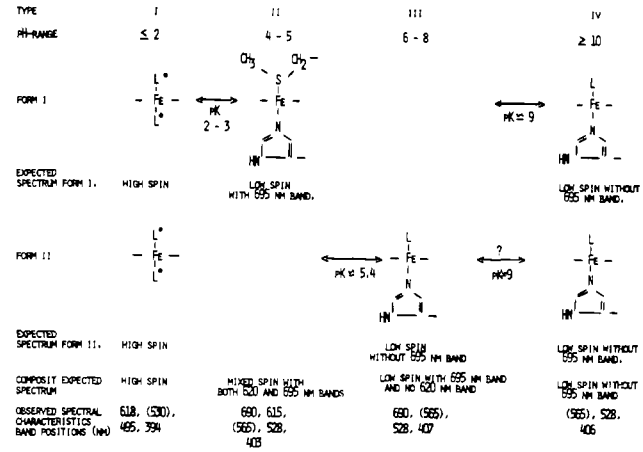

FIGURE 6: Schematic representation of the coordination configuration of heme iron of the two molecular forms in NBS–ferricytochrome *c* as a function of pH, including the expected spectral characteristics of each form, of the mixture, and of those observed for the modified preparatons. L stands for a strong intrinsic ligand-field group, and L* represents a weak ligand-field functional group.

tion. Form I undergoes transformation to a low-spin form with a p*K* of about 3, and is associated with the coordination of the sulfur of methionyl residue 80, as is the case with the native molecule (Figure 2). The relatively lower magnitude of the 695-nm band, about one-half that of the native protein, is not surprising, in view of the recently reported results from spectral perturbation studies of the native protein by Sreenathan and Taylor (1971), which showed that although the 695-nm band is ligand specific, its intensity can be diminished by conformational changes of the protein, and in view of the results from conformational studies reported in the preceding article (Myer, 1972b). The low-spin form I finally changes to another low-spin configuration with a p*K* of about 9, in the same fashion as the native molecule. The high-spin configuration of form II at low pH changes to a distinctly low-spin configuration at neutral pH's corresponding to type III with a p*K* of about 5.4. The coordination configuration of heme iron for this low-spin form is different from that of either the native protein or form I, as there is no indication of sulfur–iron coordination linkage. This is in accordance with the structure assigned to this form, *i.e.*, one containing additional modification of methionyl residue 80 to sulfoxide, which presumably inhibits sulfur coordination. The low-spin configuration assigned to form II, without sulfur coordination at one of the two positions of heme iron, is consistent with the findings that the simple heme–peptide isolated from the native molecule does bind methionine sulfoxide and that the complex exhibits a low-spin spectrum in spite of the unavailability of sulfur for coordination (O'Brien, 1969). The coordination of the oxygen atom of methionine sulfoxide or of another strong ligand-field group, possibly the imidazole of histidine, could easily account for the observed spectral properties of this form. In this regard, it is of note that the ratio of extinction of the α to β bands for terminal form II, *i.e.*, the 9:1 NBS-modified preparation, is almost the same as that observed for heme–octapeptide (a heme fragment isolated from the native protein by pepsin and trypsin degradation) in the presence of imidazole (Myer and Harbury, 1972; Harbury and Loach, 1960), in which the coordination configuration is the imidazole of histidyl residue 18 and extrinsic imidazole at positions 5 and 6 of heme iron. It is also notable that a proton-linked function, centered in the pH region 5–6 and associated with the transformation of the high-spin configuration to a low-spin form lacking the 695-nm band, has been observed in almost all chemically modified preparations of this protein in which the apparent effect is the blocking of the sulfur of methionyl residue 80 (Schejter *et al.*, 1969, 1970; Schechter and Saludjian, 1967; Stellwagen, 1968). The question of whether the transformation of high-spin form II to the low-spin form occurs with a single p*K* of 5.4 or by two distinct proton-linked functions could not be answered from these studies. Finally, since both the 3:1 and 6:1 NBS-modified preparations exhibit spectra very similar to that of the native protein in type IV, whereas the spectra of types II and III differ from the native protein, it seems that there is an additional p*K* at about 9 in the case of form II as well. In the reduced state of the metal atom, both forms of NBS-modified cytochromes are in a low-spin configuration of the metal atom. Since form I contains unaltered methionyl residue 80, and since the spectral characteristics of form I in the visible region of the spectrum (reflecting the coordination configuration of the heme iron) are more or less indistinguishable from those of the native protein, it is safe to assume that the coordination configuration of form I in the Fe^{2+} state is identical with that of the native protein. From a similar rationale, it is evident that the coordination configuration of form II in the reduced state is an altered configuration, possibly the histidyl side chain of residue 18 and a strong intrinsic ligand-field group other than methionine sulfur at position 6 of the metal atom. The definitive confirmation of these structure–function relationships, however, requires the isolation of the two forms and investigation of their physicochemical properties. These and other aspects are presently being investigated.

Regarding the activity of the NBS-modified cytochromes, the loss of the NADH–cytochrome *c* reductase activity follows the profile of the reaction corresponding to the first step of the reaction, *i.e.*, the formation of form I; and the reduction of the succinate oxidase activity occurs, in a consecutive

manner, and extends over the entire reaction range investigated; it parallels the formation of form II (Myer, 1972a). The 3:1 NBS-modified preparation exhibits minimal reductase activity, whereas the succinate activity is lowered by about 30% only (Table I). The estimate of the amount of form II in the 3:1 NBS-modified preparation is of the order of 30–40%, which agrees rather well with the proportion of reduction of the succinate activity of the protein (Table III). Similar agreement exists between the estimated proportions of form II and the residual oxidase activity of the protein for the other two preparations as well (Tables I and III). Thus, consistent with earlier conclusions (Myer, 1972a), the NBS form (form I) with modified tryptophan-59 and methionine-65 and with unaltered, but definitely perturbed, central coordination configuration is the one with intact succinate oxidase activity and reduced NADH–cytochrome *c* reductase activity. The form with additional modification of methionine-80 and with structurally changed coordination configuration, form II, is the one which lacks both NADH–cytochrome *c* reductase activity and succinate oxidase activity. The latter is consistent with the well-accepted view that the replacement of methionyl sulfur from position 6 of heme iron results in elimination of the electron-transport property of the molecule. Detailed investigations of the purified preparations are being conducted to ascertain the two-function, two-structure relationships which emerge from these studies.

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Isolation and Purification of Histones from Avian Erythrocytes†

Lee A. Sanders*‡ and Kenneth S. McCarty

ABSTRACT: The six major histones present in avian erythrocytes were isolated and purified by a combination of techniques including selective extraction, oxidation, gel filtration on Sephadex G-100, and ion-exchange chromatography on Bio-Rex 70. The histones were identified by polyacrylamide gel electrophoresis and amino acid analysis. Amino acid compositions of the purified histone fractions are quite similar to corresponding fractions reported for calf thymus tissue. Histone F3, the only cysteine-containing histone, appears to be extracted exclusively in the monomer form; the dimerization of F3 under oxidative conditions facilitates its separation from the other histones by means of gel filtration. The purification procedure reported here has proven to be equally effective for the isolation of histones from avian reticulocytes as well as mature erythrocytes.

The histones isolated from a number of sources appear to be quite similar from species to species in terms of molecular size, charge, and even primary structure (Crampton *et al.*, 1957; DeLange *et al.*, 1969; Panyim *et al.*, 1971; DeLange and Smith, 1971). Many earlier procedures for histone fractionation and characterization have been recently reviewed by Hnilica (1967), Butler *et al.* (1968), Johns (1971), and

† From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. *Received April 6, 1972.* This work was supported in part by Grant 5R01-GM-12805 from National Institutes of Health.

‡ M.D.–Ph.D. candidate, trainee of the National Institutes of Health, National Institute of General Medical Sciences (T5-GM-01678).