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Structure and Enzymatic Properties of *N*-Bromosuccinimide-Modified Horse Heart Cytochrome *c*†

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ABSTRACT: Horse heart ferricytochrome *c*, when treated with incremental additions of *N*-bromosuccinimide (NBS) at pH 4.1, undergoes modification of the invariant tryptophanyl, both methionyl and two of the four tyrosyl residues. The relative susceptibilities of the methionyl and tryptophanyl side chains depend upon the purity of the reagent, the temperature of the reaction, and the pH of the solution. The spectroscopic analysis of the reaction and comparison of the results from amino acid analysis show that in general the NBS reaction initiates with the oxidation of the single indole moiety, one of the methionyl residues, and oxidative bromination of one of the four tyrosyl side chains. The modification of the tryptophanyl and the first of the methionyl side chains is completed at about a 3.3:1 NBS/protein ratio, whereas the modification of tyrosine reaches completion at about a 5:1 NBS/protein ratio. Following the first type of structural alterations, the second methionyl residue is slowly oxidized, which reaches completion at about an 8–9 NBS/protein ratio. In addition, a second tyrosyl side chain is modified, but in a rather narrow range of reagent/protein ratios, *i.e.*, between 5 and 7. The CNBr cleavage of the NBS-modified preparation containing about 1.6 modified methionyl residue to methionine sulfoxide releases a peptide which conforms to the amino acid composition expected for a peptide resulting from cleavage at methionine-80. The first methionine to be attacked by NBS therefore is residue 65, while the one with lower susceptibility to oxidation—*i.e.*, at higher NBS/protein ratios—is methionine-80. Concurrent with the first spectroscopic step there occurs a continuous decrease of NADH–cytochrome *c* reductase activity, whereas the alteration of the succinate oxidase activity is centered in the latter stages of the reaction. The NADH activity and the tryptophanyl content bear a direct 1:1 relationship. The activity determined with the succinate oxidase system, on the other hand, seems to be governed by the integrity of methionine-80, the centrally coordinating group of the molecule. Possible structural–functional differentials for the electron capture and donor characteristics of the enzyme are suggested.

All functionally homologous mammalian-type cytochromes *c* contain a single invariant tryptophanyl residue, 59 in the vertebrate numbering system (Margoliash and Schejter, 1966). The elucidation of the physicochemical and functional significance of this consistent structural feature has been of interest to many laboratories. The commonly used approach has been modification of the functional group with group-specific reagent, and correlation of the physicochemical characteristics of the modified preparation with those exhibited by the native enzyme. Strittmatter and Ball (1954) first reported that the reaction of ferricytochrome *c* with *N*-bromosuccinimide (NBS)[1] results in an autoxidizable product with altered biological function. Detailed spectroscopic investigations of a single NBS-modified preparation (Stellwagen and Van Rooyan, 1967; Yonetani, 1968) lead to the suggestion that the spectral changes of the molecule upon reaction with NBS are reflections of tryptophan modification. Based on comparative spectral studies of the NBS-modified preparation and simple model systems, O'Brien (1966, 1969) suggested that the spectral alterations resulting from NBS treatment are due to the replacement of the methionine-coordinating sulfur in the native molecule (Harbury *et al.*, 1965; Schechter and Saludjian, 1967) with the oxygen of the methionine sulfoxide resulting from the NBS reaction. The investigations of the functional significance of the invariant tryptophanyl side chain thus far undertaken have been limited to the investigation of only a single product, with little or no struc-

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

tural characterization. In this communication we wish to report detailed results of the NBS reaction with horse heart ferricytochrome *c*, including the structural characterization of the modified preparation, and the implications of the structural alterations on both aspects of electron-transport function in the molecule. In the accompanying articles are reported the various physical properties of the NBS-modified preparations, including results from conformational studies based on circular dichroism spectroscopy.

Materials and Methods

Commercial horse heart cytochrome *c* (type III) was obtained from Sigma Chemical Co. and was used without any further purification. Crystalline horse heart cytochrome *c* (a gift from Dr. E. Margoliash) was used to check the constancy of results obtained with the commercial preparation. *N*-Bromosuccinimide, bromoacetic acid, and cyanogen bromide were repeatedly crystallized prior to use. NADH and NADH–cytochrome *c* reductase were purchased from Sigma Chemical Co. and used without any further purification. All other chemicals used were of the highest grade available.

NBS Reaction. Solutions (10–50 ml) of ferricytochrome *c* containing 0.1–4 mg/ml of protein in 0.1 M acetate buffer (pH 4.1) were reacted with NBS by addition of incremental volumes of the reagent solution. The reaction was performed in a specially designed apparatus which allows simultaneous measurement of the absorption spectrum and pH and permits the addition of known volumes of the reagent through a Gilmont ultraprecision syringe under anaerobic and thermostatic environment (Y. P. Myer and H. A. Harbury, to be published). The reaction solutions were thoroughly flushed with ultrapure helium prior to addition of reagent, and the helium atmosphere was maintained during the reaction. The absorption spectrum was recorded on a Cary 15 and/or a Bausch and Lomb Spectronic 505 spectrophotometer, and the pH measurements were conducted with a Radiometer Model 26 pH meter. The modified preparations were recovered first by desalting by Sephadex G-10 column chromatography (1.5 × 40 cm; effluent 0.1 M NH_4HCO_3) followed by repeated flash evaporation and lyophilization.

Amino Acid Analysis. Samples of the native and the modified preparations were hydrolyzed with 6 N HCl at 110° for 22 hr under vacuum. Performic acid oxidation was done according to the procedure outlined by Hirs (1956). The analysis was performed on a self-assembled amino acid analyzer using a Bio-Rad resin (Aminex 6) single column with a three-buffer system. The methionine sulfoxide was estimated indirectly by using the technique described by Neuman *et al.* (1962). The procedure in essence is first the protection of the unmodified methionine residues by carboxymethylation, and then the estimation of methionine sulfoxide as methionine sulfone after performic acid oxidation. The carboxymethylation of the native and the NBS-modified preparation was performed as follows. Native or NBS-modified protein (5–8 mg) was added to 2 ml of freshly prepared bromoacetic acid solution (0.2 M) in 0.1 M acetate buffer (pH 3.0) at room temperature. After 72 hr the reaction was terminated, and simultaneously the excess of reagent and salts was removed by passing the reaction mixture through a Sephadex G-10 column equilibrated with 0.1 M ammonium bicarbonate solution. The fractions containing cytochrome *c* were pooled and subjected to repeated flash evaporation. The CM preparations were then subjected to performic acid oxidation, acid hydrolyses, and amino acid analysis. The color constant for methionine sulfone was determined by separate experiments using a performic acid oxidized standard amino acid mixture. The amount of methionine sulfone obtained for the native protein after carboxymethylation was taken as reference zero content of sulfoxide, and the results without carboxymethylation were taken as the maximum expected yield for methionine sulfone from sulfoxide.

The tryptophanyl content of the protein was estimated using the spectroscopic method suggested by Patchornik *et al.* (1960). The extent of tryptophan modified by NBS was estimated from the decrease of absorbance at 280 nm, after appropriate corrections for contributions due to tyrosine modification; the difference from one was taken as the unreacted tryptophanyl content of the proteins.

CNBr Cleavage. The native and modified proteins were cleaved with CNBr at the methionine sites by a slight modification of the procedure described by Holmgren and Reichard (1967). In general, the cleavage was performed in a solvent containing 63% HCOOH and 37% water (v/v) with a protein concentration of about 10 mg/ml and an excess of CNBr (550 M). The reaction was allowed to proceed for 20–24 hr at room temperature, after which it was terminated by the addition of 10–15 volumes of water, and the solution was lyophilized.

Biological Activity. Succinate oxidase activity was measured according to the procedure of Schneider and Potter (1943), and the NADH–cytochrome *c* reductase activity was determined by employing the procedure reported by McGowan and Stellwagen (1970). The Beckman Kintrac VII was used to follow the rate of reduction of the enzyme. Both the NADH and the NADH–cytochrome *c* reductase solutions were used within 5–7 min after preparation in order to avoid ambiguity due to rather rapid deterioration of NADH upon standing in aqueous solution. The ΔA_{550}/min per μmole for native protein after a period of 10 min of reaction was taken as 100%.

Concentration of cytochrome *c* was determined spectrophotometrically using either the extinction coefficient of 1.05×10^5 M^{-1} at 410 nm or 1.12×10^4 M^{-1} at 529 nm. The concentrations of the modified preparations, on the other hand, were determined by using an extinction coefficient of 0.658×10^4 M^{-1} at 504 nm at pH 4.1.[2]

Results

The spectral changes resulting from incremental additions of NBS to solutions of ferricytochrome *c* at pH 4.1 are shown in Figure 1, and the results are summarized in Figure 2. The reaction can be best described as being a composite of three spectroscopically distinct steps. The first step is discerned from a decrease in extinction at 280 nm reaching a minimum value at NBS/protein ratios of 3.3 to 4.5, depending upon the extent of crystallization of the reagent. The decrease is typical of the oxidation of the tryptophan moiety to the oxindole derivative, which results in a blue shift of the 280-nm peak (Green and Witkop, 1964). Paralleling the changes in the ultraviolet (uv) region, the Soret band shifts to a lower wavelength, attaining maximum absorption at about the same reagent/protein ratio as the minimum of the uv band. Similarly, the alteration at 365 nm seems to exhibit a well-defined transition, again suggesting the parallelism of the alteration with that in the uv region. The second type of spectral variations can be identified as those occurring in the region of the

[2] 504 nm is the position of the isosbestic point of the NBS reaction at pH 4.1 (see Figure 1).

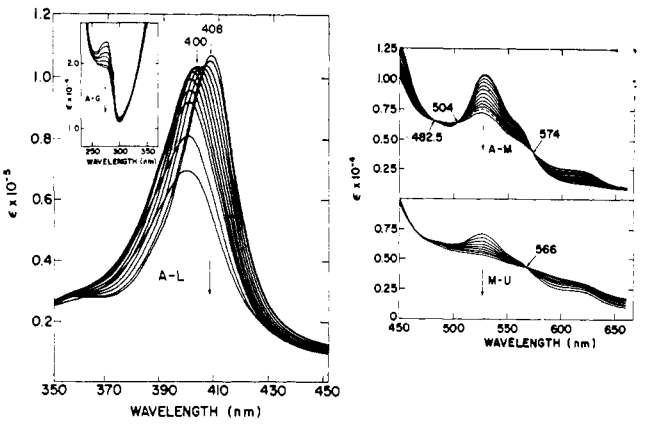

FIGURE 1: Absorption spectra of horse heart ferricytochrome *c* as a function of increasing *N*-bromosuccinimide/protein ratios. Sodium acetate buffer, 0.1 M, pH 4.01; temperature, 22°. *N*-Bromosuccinimide added in incremental amounts; five-times crystallized from water. NBS/protein ratios: Visible region: (A) 0.0, (B) 0.49, (C) 0.99, (D) 1.49, (E) 1.99, (F) 2.48, (G) 2.98, (H) 3.48, (I) 3.97, (J) 4.47, (K) 4.97, (L) 5.46, (M) 5.96, (N) 6.95, (O) 7.95, (P) 8.94, (Q) 9.93, (R) 10.93, (S) 11.92, (T) 12.92, and (U) 13.40. Soret region: (A) 0.0, (B) 0.64, (C) 1.28, (D) 1.92, (E) 2.52, (F) 3.20, (G) 3.83, (H) 4.47, (I) 5.11, (J) 5.75, (K) 7.35, and (L) 8.94. Ultraviolet region: (A) 0.0, (B) 0.51, (C) 1.04, (D) 1.50, (E) 2.00, (F) 2.50, and (G) 3.00.

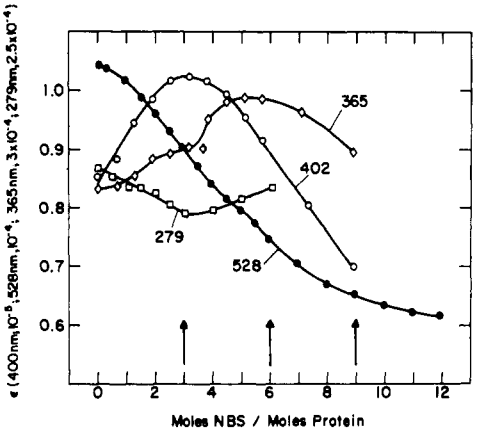

FIGURE 2: Effect on the extinction at various extrema in the absorption spectra of horse heart ferricytochrome *c* with increasing *N*-bromosuccinimide/protein ratios. Sodium acetate buffer, 0.1 M, pH 4.01; temperature, 22°.

528-nm band, which reflects predominantly the contributions from ligand–porphyrin transitions. The variations in this region, however, become evident at a slightly higher NBS/protein ratio than those classified as type I (Figure 2). Furthermore, these changes seem to continue over the entire reaction range investigated. The presence of a slight alteration in the general form of the changes at 528 nm in the region of 5–7 NBS/protein ratios, which is further discerned by the changes of the isosbestic points (Figure 1), and the presence of a second well-defined spectral inflection in the titration curve at 365 nm, possibly indicate a third type of spectral alteration. This may be a result of structural alterations of the molecule different from those of either the first or the second type of changes. There undoubtedly is significant overlap in the spectral variations between the first and the second types, but the presence of well-defined characteristics in the spectral titration curves indicates that the various structural changes of the molecule do indeed occur in a definite sequential manner.

The amount of reagent required to bring about the inflection for the spectral changes at 280 nm is dependent both on the pH of the reaction as well as on the purity of the reagent. The titration of ferricytochrome *c* with once-crystallized NBS exhibited a minimum at about 4.5 NBS/protein ratio (Table I), while with five-times-crystallized NBS the minimum occurred at about a 3.3 NBS/protein ratio. The former observation is similar to that reported by Stellwagen and Van Rooyan (1967). In addition, when once-crystallized NBS was used, the spectroscopic steps were poorly resolved as compared to five-times-crystallized reagent (Figures 1 and 2). The spectral alterations in the visible region were devoid of well-defined isosbestic points; the changes at 365 nm did not show separation of the two steps (Figure 2), and the correspondence between the inflections at the 402- and the 365-nm bands was lacking (Figure 2). The purity of the reagent therefore seems to govern the nature of the reaction, especially in determining the selectivity of the changes resulting in resolution of spectral alterations in distinct steps.

The chemical characterization of the NBS-modified preparation based on amino acid analysis shows that in addition to the modification of the tryptophanyl side chain, there occurs chemical modification of both the methionyl residues and of at least two of the four tyrosyl side chains as well (Figure 3). The methionyl side chains are modified to sulfoxides (Stellwagen and Van Rooyan, 1967; O'Brien, 1966), and the tyrosyl side chains are possibly oxidatively brominated (Schmir and Cohen, 1961). Paralleling the modification of the single tryptophanyl side chain, one of the two methionyl side chains appears to be oxidized, whereas the loss of the first of the tyrosyl side chains reaches its limiting value at about a 5:1 NBS/protein ratio. The second methionyl side chain is altered rather slowly with the alteration extending

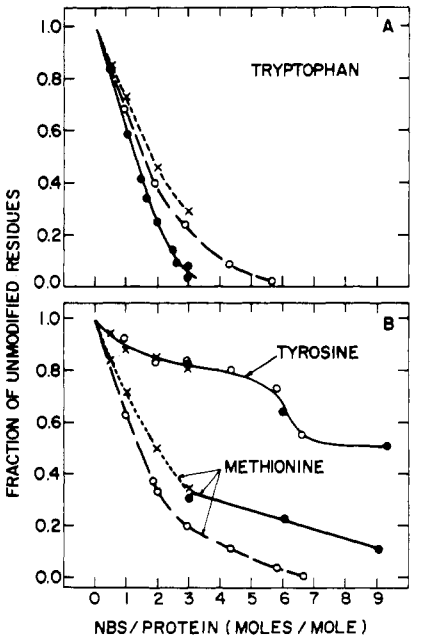

FIGURE 3: Extent of modification of various functional groups of horse heart ferri cytochrome *c* with increasing *N*-bromosuccinimide/protein ratios at two different temperatures and using once- and five-times-crystallized reagent. Buffer 0.1 M acetate, pH 4.01. (○) Once-crystallized NBS and at 22°; (●) five-times-crystallized NBS and at 22°; (×) once-crystallized NBS and at 1°. Fraction of modified residues based on one tryptophan, four tyrosines, and two methionines.

TABLE I: Structural and Functional Characteristics of NBS Preparations.

	Native	1	2	3	4	5	6	7	8	9	10	11	12	13
Reaction Conditions Other Than pH 4.1 and 0.1 M Acetate		Temperature 22° Once Crystallized						Temperature 1° Once Crystallized				Temperature 22° Five-Times Crystallized		
Purity of Reagent:														
NBS Equiv for Minimum OD at 280 nm:		4.5						3.8				3.3[a]		
Preparation:														
NBS/Protein Ratio:		0.97	1.94	2.90	4.36	5.81	6.60	0.51	1.02	2.01	3.03	3.01	6.02	9.00
Amino Acid Content	Residues/Mole													
Tryptophan[b]	1.0	0.68	0.40	0.24	0.06	0.02	0.00	0.85	0.73	0.46	0.29	0.02	0.00	0.00
Methionine sulfoxide[c]	0.0	0.7	1.3		1.8	1.9	2.0	0.3	0.6	1.0	1.3	1.3	1.6	1.8
Methionine[d]	2.0	1.3	0.7		0.2	0.1	0.0	1.7	1.4	1.0	0.7	0.7	0.4	0.2
Tyrosine[e]	4.0	3.8	3.3	3.3	3.2	2.9	2.2	3.8	3.6	3.4	3.3	3.3	2.7	2.1
Histidine	3.0	2.8	2.8	2.9	3.0	2.9	3.0	2.9	3.0	3.1	3.0	3.0	2.9	2.9
Activity														
NADH–cytochrome *c* reductase activity (%)	100	72	52	40	30	27	26	90	82	68	49	28	32	26
Succinate oxidase activity (% rate at 6 μM concentration)	100					ND	ND	100	96	93	70	68		ND

[a] From results shown in Figure 1. [b] Amount of modified tryptophan estimated by spectral procedure using the expression $\Delta OD_{280} \times 1.31/5500$ (Patchornik *et al.*, 1960). The difference from one is presented as the tryptophan content. [c] Methionine sulfoxide determined indirectly as methionine sulfone after carboxymethylation and performic acid oxidation (see Results). The results from the native protein after the completion of the entire procedure was taken as zero of methionine sulfoxide, and the result after omission of the carboxymethylation step was taken as 100%. [d] Difference between the actual amount of methionine in native protein and the methionine sulfoxide content of the preparation. [e] Corrected for destruction during hydrolysis. Based on recovery of 3.6 residues in native protein. [f] Based on ΔOD_{550}/min per μmole after 10-min reaction. Native taken as 100%.

over the entire reaction range. The modification of the second tyrosyl side chain occurs in a narrow range of the reaction—*i.e.*, between 5-7 NBS/protein ratios (Figure 3). The comparison of these results to spectral changes (Figure 2) indicates a definite relationship between the nature of the structural change and the spectral alterations. The first spectroscopic step parallels the modification of the tryptophanyl side chain and the modification of at least one of the two methionyl side chains (Table I and Figure 3). The second spectral step, discerned by variation in the 528-nm band, appears to be associated with modification of the second of the methionyl side chains, since this is the only structural feature of the molecule, the modification of which extends over the entire range of the reaction (Figure 3). In addition to the modification of the methionyl and tryptophanyl residues, two of the four tyrosyl side chains are altered, but the modification of only one of the two residues seems to be correlated with the spectroscopic alteration of the molecule. The first of the tyrosyl residues undergoes modification during the initial part of the reaction—*i.e.*, it is completely modified at about 5 equiv of the reagent (Figure 3)—but no spectral transitions can be related to this aspect of the structural alterations. Within the range of the modification of the second tyrosyl residue—*i.e.*, between 5 and 7 equivalents of NBS—a definite inflection in the absorptivity at 365 nm and an inflection in the variation at 528 nm exist concurrently.

The reaction of native cytochrome *c* with cyanogen bromide results in cleavage at both methionyl peptide bonds 65 and 80 and in the release of three peptide fragments: one containing the heme moiety, from residues 1 to 65, and two from sequences 66 to 80 and 81 to 104 (McGowan and Stellwagen, 1970). The presence of methionine sulfoxide however has been shown to prohibit the reaction with CNBr (Green and Witkop, 1962), which has recently been confirmed by Jori *et al.* (1970). If the two methionyl residues are being modified in a sequential manner in the NBS reaction, then the cleavage of the preparation containing more than one modified residue should yield a single heme–peptide fragment due to cleavage at the unreacted residue; otherwise, one should obtain a mixture of heme–peptide fragments corresponding to cleavage at both the susceptible bonds in the molecule. The chromatographic elution patterns resulting from CNBr cleavage of the native and the 6:1 NBS-modified preparation, which contains about 1.6 modified methionyl residues (Table I), are shown in Figure 4, and the amino acid compositions of the various fractions are compared in Table II. Only one type of heme-containing fragment was detected, the amino acid composition of which corresponds to the fragment resulting from cleavage at methionyl residue 80. Thus the cleavage of the 6:1 NBS preparation is limited to the methionine-80 peptide bond, and there seems to be no detectable cleavage at position 65 of the molecule. The unmodified methionyl side chain in this preparation evidently is residue 80, and therefore the slowly oxidizing methionyl side chain is residue 80. Thus the residue undergoing oxidation during the early stages of the NBS reaction, paralleling the alteration of the tryptophanyl side chain, is methionyl residue 65.

TABLE II: Amino Acid Analysis of Native, NBS-Modified (6:1) Preparation, and of Various Fractions after CNBr Cleavage.[a]

Amino Acid	Native	NBS Prep	Fractions after CNBr Hydrolysis and Chromatographic Separation					
			Native		NBS-Modified Preparation			
			C_1 1–65	C_2 66–80	A_1	B_1	B_2	B_3 1–80
Asp	8.0 (8)	7.9 (8)	5.3 (5)	1.0 (1)	8.2	8.0	7.8	5.9 (6)
Thr	9.2 (10)	9.3	6.4 (7)	0.9 (1)	8.7	8.9	10.4	7.8 (8)
Ser	0 (0)	0	0 (0)	0 (0)	0	0	0	0
Glu	12.0 (12)	12.1	6.9 (7)	1.7 (2)	11.9	11.5	12.0	9.4 (9)
Pro	ND (4)	ND	1.8 (2)	1.9 (2)	ND	ND	ND	ND
Gly	12.2 (12)	12.1	ND (10)	0.8 (1)	11.7	11.5	11.8	10.7 (11)
Ala	6.1 (6)	5.7	3.0 (3)	0.2 (0)	5.8	6.2	6.0	2.9 (3)
Cys	1.7 (2)	1.6	1.6 (2)	0 (0)	ND	ND	ND	ND (2)
Val	3.0 (3)	2.8	2.3 (3)	0.1 (0)	3.0	3.2	3.1	3.0 (3)
Met	1.8 (2)	0.6	0 (0)	0 (0)	1.6	1.6	1.9	0.6 (1)
Ile	5.8 (6)	6.1	1.8 (2)	1.4 (1)	5.6	5.2	6.3	3.1 (3)
Leu	5.9 (6)	5.8	3.1 (3)	0.8 (1)	5.4	5.5	5.8	4.2 (4)
Tyr	3.6 (3)	2.4	0.9 (1)	0.9 (2)	1.42	1.7	3.1	1.6 (3)
Phe	3.7 (4)	3.6	2.6 (3)	0.1 (0)	3.6	3.6	4.1	2.7 (3)
His	2.9 (3)	2.8	2.8 (3)	0 (0)	2.8	2.9	2.8	2.8 (3)
Lys	18.9 (19)	18.2	11.3 (11)	2.8 (3)	18.8	19.3	18.7	13.7 (14)
Arg	1.9 (2)	1.8	ND (1)	ND (0)	ND	1.9	ND	ND

[a] Values in parentheses are residues per molecule.

The heme fragment 1–80 contains three of the four tyrosyl residues, 48, 67, and 74. The heme–peptide released from the 6:1 NBS-modified preparation contains 1.8 unmodified tyrosyl residues (Table II), whereas the preparation itself had about 2.7 unmodified residues (Table I). The tyrosyl side chain undergoing alteration during the first part of the reaction therefore is one of the three tyrosines, 48, 67, and 74. The tyrosyl side chain under attack by NBS is insensitive to both the purity of the reagent and the temperature of the reaction (Figure 3), and the results from spectroscopic titration of the phenolic groups in the preparation containing about 0.7 modified tyrosyl side chain (Table I, preparation 11) show that the tyrosyl residue affected during the early stages of the NBS reaction is one with an apparent pK of 10.2 in the native protein (see following article, Myer and Pal, 1972). This suggests that the susceptible tyrosyl side chain is one of those exposed to the solvent. If this is the case, then it is expected that the modification of this functional group will not cause any detectable spectral alteration during the course of NBS reaction. As inferred in the preceding paragraphs, this indeed seems to be the case. Based on X-ray diffraction data (Dickerson *et al.*, 1971a), of the three possibilities, 48, 67, and 74, residue 74 appears to be the one. Confirmation of this assignment and determination of the structural location of the second NBS-susceptible tyrosyl residue is the subject of continued investigations.

In addition to the tryptophanyl, methionyl, and tyrosyl residues, the reaction of NBS with proteins has been shown to result in the oxidation of the imidazole moieties of the histidyl side chains (Schmir and Cohen, 1965), as well as cleavage of the polypeptide chain at the susceptible sites (Witkop, 1961). The possibility that the histidyl residues are oxidized by the NBS reaction was ruled out, since the results from acid hydrolysis failed to provide any indication of decrease in the histidine content of the preparations (Table I). Similarly, the possibility of peptide-bond cleavage, if any, in the range of the reaction investigated was eliminated, as attempts to identify the terminal amino acid, resulting from cleavage by employing the dinitrophenylation procedure as described by Sanger (1949) followed by identification of the Dnp-amino acids as described by Fraenkel-Conrat (1958), failed to yield positive evidence.

Enzymatic Activity. The unique location of cytochrome *c* in the electron-transport chain allows the differentiation of its electron-transport biological function into two aspects, the electron capture or reducibility, and electron donation or oxidizability. The former can be measured by its rate of reduction by NADH–cytochrome *c* reductase, and the latter by the enhancement of the rate of oxygen consumption in

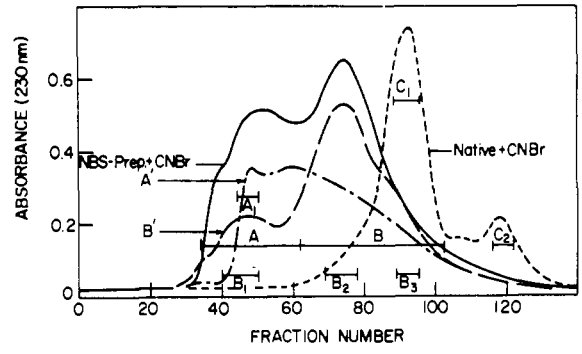

FIGURE 4: Exclusion chromatography of CNBr-hydrolyzed native horse heart ferricytochrome *c* and the 6:1 NBS/protein preparation. Column: Sephadex G-75, 0.9 × 125 cm, rate of flow, 5 ml/hr; temperature, 22°; effluent, 0.1 M ammonium bicarbonate. Fractions containing 1.5 ml were collected. Pooled fractions C_1 and C_2 refer to those from the native protein, and A and B to those from the NBS-modified preparation. The exclusion profiles labeled A′ and B′ are the rechromatographed profiles for fractions A and B, respectively. Fractions with subscripts are those with amino acid analyses given in Table II.

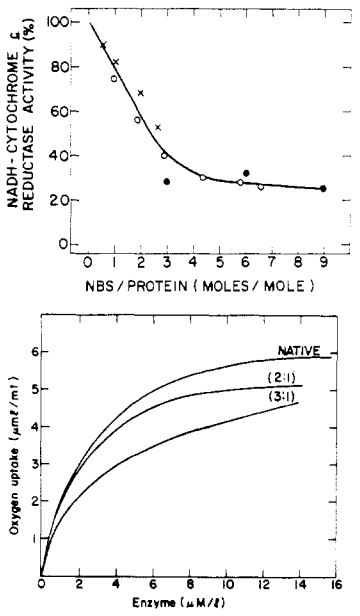

FIGURE 5: NADH–cytochrome *c* reductase and succinate oxidase activity of various NBS-modified horse heart ferricytochrome *c* preparations. (A) NADH–cytochrome *c* reductase activity. Conditions: 0.08–0.14 μmole of protein, 1.31 μmoles of NADH, 40 μmoles of potassium phosphate (pH 8.5) at 22°. Total volume 1.0 ml. Reaction initiated by addition of 20 μg of reductase; absorption measured at 550 nm. ΔA_{550}/min per μmole of protein after 5- and 10-min reaction were compared to the native protein. The fractional activity is expressed as per cent activity. (B) Succinate oxidase activity. Assay conditions; total volume 3 ml containing 3.1×10^{-2} M phosphate buffer (pH 7.4), 4×10^{-4} M $AlCl_3$, 3.9×10^{-4} M $CaCl_2$, 5×10^{-2} M sodium succinate, 15 mg of fresh rat liver homogenate in water. Central well contained 0.2 ml of 2 M sodium hydroxide. NBS preparations used were those prepared at room temperature with once-crystallized NBS.

the succinate oxidase system. The results for both are shown in Figure 5A,B, respectively. The reducibility of the protein decreases continuously with increasing modification with NBS and reaches a minimal value of about 30% in the case of preparations at about 3.5 equiv of the reagent (Figure 5A). The lowering of the reducibility seems to begin at the onset of of the reaction; furthermore, minimal activity is maintained over all higher regions of the reaction range investigated. The oxidizability property, on the other hand, is more or less unaffected during the early stages of the reaction (Table I, preparations 7 and 8). The first detectable lowering of oxidizability occurs in preparations at about 2 equiv of the reagent (Table I, preparation 9). With further modification of the protein, however, there is a continuous lowering of this biological function of the molecule. It is only in cases of preparations at the highest limits of the reaction—*i.e.*, at a 9:1 NBS/protein ratio—that the enhancement of the oxygen uptake becomes undetectable. Thus, in addition to the reducibility of the protein, the oxidizability property is also altered by NBS modification, but its variation is centered in a different region of the reaction range, and furthermore, it extends over the entire range investigated. The modification of the two aspects of the biological function therefore occurs in two distinct and consecutive steps, the first localized in the early part of the reaction and reaching minimal values at about the same point as the first spectroscopic step, and the second occurring after a lag and extending over the entire range of the reaction. The second spectroscopic step seems to concur with the profile of the oxidizability function of the protein. Since different structures of the molecule are attacked at different stages of the NBS reaction, and since the variation of different structures is reflected in differentiation of the spectroscopic alterations into distinct steps, it seems the loss of the two aspects of the activity must be connected with different structures of the protein.

Discussion

N-Bromosuccinimide is one of the well-known and widely used reagents for the determination of the tryptophanyl content of proteins, presumably because of its selective reactivity toward the indole moiety of this residue. During the past few years it has become evident that in addition to modification of the tryptophanyl side chains, NBS also attacks other functional groups in proteins, such as tyrosines, imidazole, and the methionyl side chains. It is known to cleave both the tryptophanyl and the tyrosyl bonds in proteins as well as in peptides (Witkop, 1961). Horse heart cytochrome *c*, in addition to the susceptible tryptophanyl residue at position 59, contains four tyrosyl residues at positions 48, 67, 74, and 97, two methionyl residues at positions 65 and 80, and three histidyl residues at positions 18, 26, and 33 (Margoliash and Schejter, 1966). The exposure of horse heart ferricytochrome *c* to incremental amounts of NBS results in attack of not only the tryptophanyl residue, but also of two of the four tyrosyl and both methionyl residues of the molecule. The relative susceptibilities and the reactivities of both the methionyl and the tryptophanyl side chains are dependent upon the repeated crystallization of the reagent, the pH of the solution, and the temperature of the reaction. The NBS reaction toward cytochrome *c* therefore seems to be rather complex and nonspecific (Stellwagen and Van Rooyan, 1967). The analysis of the spectroscopic variations with highly purified reagent, and the chemical characterization of the products, however, clearly show that the modification of various functional groups of the protein occurs in distinct steps (Figures 2 and 3 and Table I). Thus, although modification occurs at multiple sites, the functional groups still show appreciable differences in their susceptibilities toward NBS so as to warrant resolution of functional relationships of various structures of the molecule. In addition, preparations containing proportionally different extents of modification of the two functional groups can be obtained (for example, see the following sets of preparations: Table I, 2, 10 and 11; 2 and 9; 5, 6, 11, 12, and 13) by use of the fact that the susceptibilities of the methionyl and tryptophanyl side chains exhibit opposite dependence on the purity of the reagent—*i.e.*, with increasing purification there is greater modification of the tryptophanyl side chains and a corresponding lowering of the extent of methionyl modification (Figure 3 and Table I)—and by use of the fact that the lowering of temperature results in lowering of modification of both functional groups. It is this aspect of the NBS reaction which provides the appropriate molecular forms significant in the resolution of the fundamental question concerning the functional role of the various structures of the molecule. In view of the above considerations, it should be pointed out that any conclusions based on a single NBS modification (Stellwagen and Van Rooyan, 1967; Ulmer, 1966) or on preparations without appropriate control of the purity of the reagent and the temperature of the solution (Yonetani, 1968) should be reevaluated.

The structural characterization of the NBS products and the analysis of the spectroscopic alterations resulting with incremental addition of NBS indicate that the sequence of

events can best be expressed as follows. At the outset of the addition of NBS, the single indole moiety of the tryptophanyl residue is oxidized to the corresponding oxindole derivative, and methionyl residue 65 to sulfoxide. The modification of both structures is more or less complete at about 3.5 equiv of the reagent, which represents the first step of the spectral alterations. Concurrent with these structural alterations, one of the four tyrosyl residues is modified, possibly residue 74, but with completion at about 4–5 equiv of the reagent. Following one or more of these structural changes, the second methionyl residue, the centrally coordinated residue 80, is modified. Since the alteration profile of this structure (Table I and Figure 3) parallels the spectral changes ascribed to the second step (Figure 2), it seems that the two are counterparts of one another. In addition to these changes, and in a narrow range of NBS concentration, 5–7 equiv, the second tyrosyl residue is also modified, the spectroscopic counterpart of which presumably is the poorly defined third step.

From these observations, it seems that the spectroscopic properties of the NBS products can be broadly interpreted as a reflection of the formation of two distinct molecular forms in a consecutive manner. The first molecular form is reflected primarily by spectral changes designated as the first step, and the second, by the changes observed at the 528-nm band and identified as the second step. Structurally, form I contains modified methionine-65, the tryptophan-59 side chains, and possibly tyrosine-74, and form II contains additional modification of methionyl residue 80. The details of spectroscopic structural relationships (Myer and Pal, 1972) support these suggestions.

Of the various functional groups susceptible to NBS modification, the modification of the variant methionyl residue 65 (Margoliash and Schejter, 1966) and of the first tyrosyl residue, if residue 74, is consistent with the expected behavior of the two functional groups as determined from studies with other reagents (McGowan and Stellwagen, 1970; Stellwagen, 1968) and the fact that both are exposed in the native protein (Dickerson *et al.*, 1971a). The reactivity of the tryptophanyl side chain and of methionyl residue 80, on the other hand, could not merely be due to their availability, since the evidence thus far clearly shows that both these groups are not only buried (Stellwagen and Van Rooyan, 1967; Stellwagen, 1968), but also configurationally localized, either through hydrogen bonding to other protein structures (tryptophan–propionic acid side chain) or by providing the centrally coordinating group to the heme iron, *i.e.*, the sulfur of methionyl residue 80 (Dickerson *et al.*, 1971a). The lag between the spectral changes indicating the modification of methionyl residue 80 and those reflecting the structural alterations in the first part of the reaction (see Results) clearly shows that the reactivity of this functional group is due to the conformational changes resulting from preceding chemical alteration of the molecule, since under normal conditions it would not be expected to react with the reagent. This is consistent with both the conclusions based on the analysis of the spectroscopic properties of the NBS preparations (Myer and Pal, 1972) as well as the conformational studies (Myer, 1972). Based on an identical line of reasoning, the reactivity of the second tyrosyl residue toward NBS can also be adequately explained (see Results). In the case of the tryptophanyl side-chain reactivity toward NBS, there seem to be two possible explanations. (i) Under experimental conditions there is an equilibrium between the native and partially unfolded protein, and it is in the case of the latter that the functional group is accessible to the reagent, thus undergoing modification; (ii) the apparent reactivity of the tryptophanyl side chain is because of conformational alteration of the protein resulting from other structural changes in the protein, such as of methionine-65 or of the first of the tyrosyl side chains. Of the two possibilities we favor the latter, since (i) the physical characterization of the native protein as a function of pH by diversified techniques such as optical rotatory dispersion (Myer and Harbury, 1966), circular dichroism (Myer, 1968a,b), solvent perturbation (Stellwagen and Van Rooyan, 1967), and reduced viscosity measurements (Bull and Breese, 1966) have thus far failed to provide convincing evidence suggesting the occurrence of conformational alteration at pH 4, and (ii) the observation that the susceptibilities of both the tryptophanyl and methionyl side chains are altered in identical fashion both by the lowering of temperature and by a rise in pH (Figure 3) is inconsistent with the behavior expected of a buried and an exposed residue, if the reactivity of the former is due to conformational equilibrium between native and deranged forms of the molecule. The chemical reactivity of the buried tryptophanyl residue toward NBS under experimental conditions therefore must be due to conformational changes caused by chemical modification of other structures of the molecule. The two structures undergoing alteration parallel to that of tryptophan are methionine-65 and one of the tyrosyl residues, possibly residue 74. The possibility that the modification of the tyrosyl residue determines the reactivity of the tryptophanyl side chain can be ruled out, since its own susceptibility is almost independent of both the purity of the reagent as well as the temperature of the reaction (Figure 3), whereas the reactivity of the tryptophanyl side chain is dependent upon both of these parameters. Second, the limiting NBS concentration for complete modification of this structure is higher than that for the tryptophanyl side chain (Figure 3), which would not be the case if the modification of the tyrosyl side chain preceded that of tryptophan, a behavior essential for two consecutive processes. Thus the explanation for the reactivity of buried tryptophan-59 lies in the modification of the methionine-65 side chain. It means that methionine-65, though a variant feature of the protein (Margoliash and Schejter, 1966), must be involved in some fashion in determining the conformation of the protein. In this regard, additional support, although only indirect, emerges from the current conformational studies of carboxymethylated ferricytochrome *c* containing substitutions specifically at the methionyl side chains in permutation with the histidyl residues (L. H. MacDonald and Y. P. Myer, to be published), indicating that the integrity of methionyl residue 65 is definitely essential in governing the refolding property of the molecule. Similarly, definite changes have also been observed for the preparation containing substitution at methionine-65 and histidine-33 in the apparent pK's of the spectral transitions, both in the acidic pK's and the phenolic pK's (Stellwagen, 1968), which may also be taken to indicate the significance of methionyl residue 65 in determining the conformation of the molecule.

The structural alterations of ferric cytochrome *c* with NBS result in loss of both aspects of the biological function of the protein, namely, the electron capture or the reducibility as determined in the NADH–cytochrome *c* reductase system, and the electron donor or the oxidizability as determined by the enhancement of the oxygen uptake in the succinate oxidase system, but the derangements of the two aspects are located in different regions of the NBS reaction, and furthermore, they seem to occur in a consecutive manner. This is discerned from the observation that the loss of reducibility

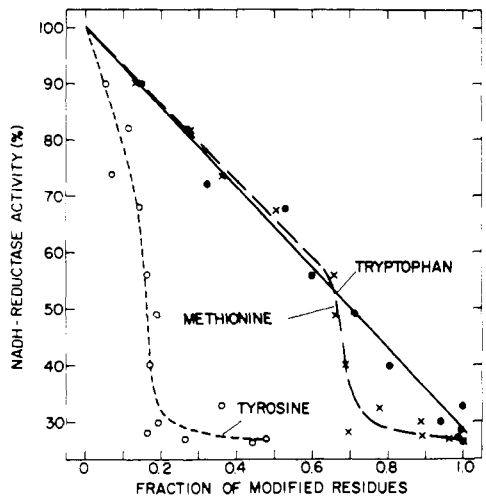

FIGURE 6: Relationship of NADH–cytochrome *c* reductase activity and the extent of chemical modification of various functional groups in horse heart ferricytochrome *c* resulting from increasing NBS modification of the protein. Fraction of the modified amino acids is based on a single tryptophan, four tyrosines, and two methionines.

occurs during the early part of the reaction and reaches a minimal value of about 30% at about 3.5 equiv of the reagent (Figure 5A), the limiting value for the first spectroscopic step. The alteration of the oxidizability characteristics of the protein, on the other hand, first becomes evident at about 2 equiv of the reagent and reaches a minimal value at the highest limit of the reaction (Table I). The loss of oxidizability parallels the second of the consecutive spectroscopic steps. Since different structures of the protein are altered in different regions of the reaction (Figures 2 and 3), the two functions must be associated with different structures of the protein. In addition, the observation that the preparations exhibiting lowered reducibility, up to 30%, show almost unchanged oxidizability properties (Table I, preparations 7, 8, and 9), and conversely, the preparations exhibiting identical oxidizability show varying proportions of the reducibility function of the protein (Table I, preparations 10 and 11), suggests that the two properties of the molecule are not directly linked with each other such that the alteration of one results in variation of the second and *vice versa*. The independent behavior of the two aspects of protein function and the dependence of each on the integrity of different structures mean that the two functions must be localized in two distinct regions of the molecule. In this regard, the hypothesis that the electron capture or the reducibility of the protein occurs through the left channel of the molecule, while the electron transfer or the oxidizability is controlled by the integrity of the structures localized in or near the vicinity of the right channel (Dickerson *et al.*, 1971b), seems to fit rather well with the above conclusions regarding both the mutual independence as well as the structural dependence of the two aspects of the biological function.

The concurrence between the loss of the oxidizability function of the protein with the second NBS spectroscopic step, which constitutes the modification of methionyl residue 80, is not unexpected, since it is well documented that both the chemical modification of the centrally coordinated amino acid side chain of methionine-80, as well as its replacement from coordination with extrinsic ligands, result in loss of biological function as determined by the succinate oxidase system (Margoliash and Schejter, 1966). In both the above cases, as well as in the case of modification with NBS, the apparent conformational effects on the molecule are the alteration of the coordination configuration of the heme iron, and significant changes in the electronic configuration of the heme iron and in the polypeptide chain (Myer, 1968a, 1972). Thus, whether the biological significance of methionyl residue 80 is because of its coordination to heme iron, the determination of the electronic configuration of the heme group, and the conformation of the polypeptide chain, or because of its direct participation in the mechanism for electron transport, cannot be deciphered from these studies or others, since it seems that all these aspects are integral constituents of the same structural aspect of the molecule, *i.e.*, the integrity of the central coordination complex (Myer, 1968b).

The linear dependence of the reducibility property of the protein on the unmodified tryptophanyl content of the NBS preparations, irrespective of the extent of modification of other structures in the protein (Figure 6 and Table I), clearly demonstrates that, of the various structures modified by this reagent, the modification of the tryptophanyl side chain results in the loss of electron capture. The functional significance of tryptophan-59 in determining the biological activity of the protein is therefore explicit. Consideration of the location of this functional group in the three-dimensional structure of the protein, and its specific configuration with respect to the heme moiety due to hydrogen bonding with one of the heme side chains (Dickerson *et al.*, 1971a), and the recently proposed mechanism for the reducibility of the protein (Dickerson *et al.*, 1971b), tempt one to suggest the direct participation of the indole moiety of tryptophan-59 in the mechanism, with the following sequence of events: reductase → some structure of the protein → tryptophan-59 → tyrosine-67 → and finally methionine-80 and heme iron. On the other hand, the conformational studies of the native protein (Myer, 1968a,b) and of NBS-modified preparations (see following article, Myer, 1972) provide convincing evidence showing that the integrity of tryptophan-59 is indeed essential in determining the electronic configuration of the heme moiety of the protein. Since the magnitude of conformational alterations resulting from the modification of tryptophan-59 are rather small, and indeed, are of a very specialized nature, the uncoupling of the electronic interactions (Myer, 1972), and since they are localized in the immediate vicinity of the heme group, it seems that the alteration of the reducibility could not be due to the inability of the molecule to accept an electron because of gross conformational alteration, but rather, because of an inability to transfer the electron to the appropriate site, namely, the heme iron. The very existence of electronic interaction between the porphyrin moiety and the indole chromophore, and its elimination upon modification with NBS, could account for the observed effects. In addition, such electronic interaction, possibly a π–π overlap, could very easily provide a pathway for the electron to reach iron, rather than the proposed scheme involving tyrosine-67, methionine-80, etc. This would indeed be consistent with the earlier conclusion that the alteration of the two functions occurs independently of each other, since it seems unlikely that a mechanism involving methionyl residue 80, which does govern the electron-donor property of the protein, would also be operational in the transfer of the electron to the metal atom.

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Circular Dichroism Studies of *N*-Bromosuccinimide-Modified Horse Heart Cytochrome *c* Preparations†

Yash P. Myer

ABSTRACT: The conformational characterization of various NBS-modified horse heart ferricytochrome *c* preparations based on circular dichroism measurements in the region 450–186 nm, thermal denaturation, effect of pH, and comparison of the Soret CD spectra to those of other modified forms containing altered central heme iron complexes—*i.e.*, the dicarboxymethylated and diiodo derivatives—has been reported. The preparations at higher than 3 equiv of the reagent, the conditions under which the oxidation of primarily methionyl residue 80 occurs, exhibit a continuous simplification of the dichroic spectra over the entire spectral region, including the intrinsic region, thus reflecting a continuous derangement of both the conformation of the protein moiety, as well as of the electronic configuration of the heme group. The preparation during the first step of the NBS reaction—*i.e.*, up to 3 equivalents of the reagent—maintains, however, most of the dichroic features of the native protein. The ellipticity at 222 nm, the negative bands at 282 and 289 nm, and the spectral details in the δ-absorption region remain unaltered, while the doubly inflected Soret spectrum of the native protein is partially simplified, and the ellipticity at the 250-nm band apparently vanishes. The denaturation profile of the terminal preparation (3:1 NBS/protein ratio) exhibits a single-step transition centered about 8° lower than the main thermal unfolding step of the native protein. The effect of pH variation between 4 and 10 is identical with that observed for native protein. The Soret spectrum of the 3:1 NBS-modified preparation differs significantly from those observed in the cases of the dicarboxymethylated and the diiodo derivatives of the protein, thus suggesting the lack of analogy between the two types of derivatives. Based on studies of CD spectra of the native molecule under different denaturation conditions, the above observations have been interpreted to indicate that the apparent conformational degradation resulting from structural alterations during the first step of the reaction is simply the uncoupling of the electronic interactions between the prosthetic group and the protein moiety with little or no change, either in the polypeptide conformation of the protein or the chemical nature of the central-coordinated complex of the molecule. A slight weakening of the iron–ligand coordination linkage is however a possibility. It is concluded that the single invariant tryptophanyl residue 59 plays a significant role in determining the conformation of the molecule.

The elucidation of structure–biological function relationships in proteins by introduction of structural alterations and investigation of chemical and functional properties of the resulting derivatives requires that the conformational implications associated with such modifications also be

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