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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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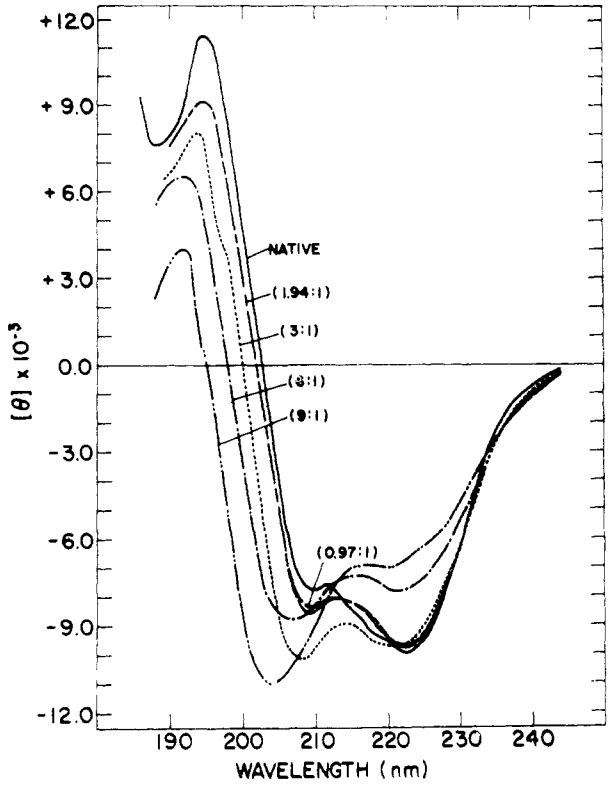

FIGURE 1: Circular dichroism spectra of native and NBS-modified horse heart ferricytochromes *c* at pH 7.0, 0.1 M phosphate buffer; temperature, 27°. 3:1, 6:1, and 9:1 preparations made with five-times-crystallized NBS at room temperature; 0.97:1 and 1.94:1 preparations made with once-crystallized NBS (see Table I of Myer (1972).

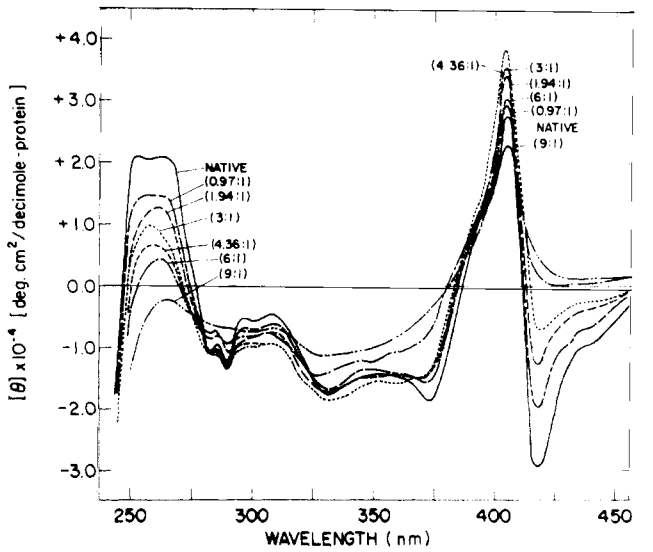

FIGURE 2: Circular dichroism spectra of native and NBS-modified horse heart ferricytochrome *c* at pH 7.0; temperature, 27°. For details of the preparation, see Table I of Myer (1972).

thoroughly investigated, since it is very likely that the resulting alterations of the biological function may not be due to changes in the direct functional participation of the structures involved, but rather, a consequence of conformational changes of the molecule. In the previous article (Myer, 1972) we reported the structure–biological function relationships resulting from modification of horse heart ferricytochrome *c* with NBS, and in this article the studies have been directed toward the understanding of the conformational implications associated with the structural alterations.

Materials and Methods

The NBS[1] derivatives used for these investigations are those whose chemical and biological–functional characteristics are reported in the preceding article (see Table I). They are identified by their respective NBS/protein ratios, *e.g.*, 3:1, 6:1, etc. The products used in these studies are primarily those obtained with five-times-crystallized NBS unless otherwise stated.

Ferricytochrome *c* was carboxymethylated at room temperature, both in the presence and absence of 0.2 M NaCN using bromoacetate (0.2 M) as the carboxymethylating reagent in accordance with the procedure used by Stellwagen (1968). Solutions containing 8–12 mg/ml of protein were allowed to react for a period of 96 hr and then desalted by Sephadex chromatography (G-50) using 0.2 M ammonium bicarbonate as the eluate. The desalted solution was repeatedly flash evaporated and finally lyophilized. The diiodo derivative of the protein was prepared by essentially the same procedure as used by McGowan and Stellwagen (1970)—*i.e.*, with an eightfold molar excess of KI_3 for 10 min at pH 9.7, except that the reaction was terminated and the solution desalted simultaneously by Sephadex chromatography, as in the case of the carboxymethylated derivatives. The carboxymethylated preparations were characterized by amino acid analysis, whereas the diiodo derivative was characterized by comparison of the $\Delta\epsilon_{243}$'s for the tyrosyl ionization of the molecule (Stellwagen, 1968; McGowan and Stellwagen, 1970).

The absorption spectroscopic measurements were made with a Cary 15 spectrophotometer, and the pH measurements with a Radiometer pH meter, Model 26. The circular dichroism (CD) measurements were conducted using a Jasco-J-10 dichrograph by the procedure already described (Myer, 1968a). The CD measurements were made in water-jacketed quartz cells with fused windows with a path length of 1 cm in the region above 250 nm and of 1 mm below this wavelength. Each observation was averaged over 5 min by using the time-averaging computer (Myer and MacDonald, 1967). The ellipticities below 240 nm are expressed as (deg cm²)/dmole of amino acid and as (deg cm²)/dmole of protein in other parts of the spectrum. The concentrations of the solutions were measured spectroscopically, using either the extinction coefficient of the appropriate preparation at 528 nm or an extinction of 0.66×10^4 M^{-1} l. cm^{-1} at 506 nm and at pH 4.1, the isosbestic point for the NBS titration (see Figure 1 of Myer, 1972).

Results

The circular dichroism spectra of various NBS-modified preparations of horse heart ferricytochrome *c* and of the native enzyme at pH 7.0 in the intrinsic absorption region are compared in Figure 1, and in Figure 2 are compared the dichroic curves in the visible and near-ultraviolet regions of the

[1] Abbreviations used are: NBS, *N*-bromosuccinimide; CM_1, monocarboxymethylated derivative of horse heart ferricytochrome *c* containing modified Met-65 and His-33; CM_2, dicarboxymethylated derivative of horse heart ferricytochrome *c* containing modified Met-65, Met-80, and His-33; DI_2, diiodo derivative of horse heart ferricytochrome *c* containing modified Tyr-67 and Tyr-74.

spectrum. The modification of the enzyme with NBS up to about a 3:1 reagent/protein ratio, the first spectroscopic step of the NBS reaction, results in a very small change in ellipticity at the 222-nm negative band, whereas the ellipticities at both the 208- and the 196-nm inflections exhibit significant deviation in their rotatory strengths from those of the native molecule. The modification of the protein with higher proportions of the reagent, on the other hand, changes all the dichroic inflections in this wavelength region. The 222-nm band is replaced by a shoulder, the 196-nm peak undergoes significant decreases in intensity, and the 208-nm inflection shifts to the blue with a significant gain in rotation. The denaturation studies of the native protein (Myer, 1968b), as well as of model polypeptide systems (Myer, 1969) and the computer-simulated curves for systems containing varying proportions of organized and unfolded structures (Greenfield and Fasman, 1969; Myer, 1969) indicate that the expected alterations in this region resulting from derangement of the polypeptide conformation should reflect a continuous decrease of ellipticity at the 222-nm band with a concurrent blue shift and decrease of ellipticity at the positive peak. Finally a new negative minimum should develop in the vicinity of 200 nm. The variations at higher than 3 equiv of NBS are very similar to those expected, whereas there are inconsistencies in the observed variations up to 3 equiv of NBS. Thus the alterations in the intrinsic absorption region up to 3 equiv of NBS cannot be interpreted with certainty as reflections of conformational derangement of the polypeptide moiety of the molecule. The alteration of contributions from transitions other than the amide bonds, especially when located below 210 nm, could easily induce the observed dichroic changes in Figure 1. In this regard, the altered contributions of one or more of the heme transitions located in this region of the spectrum (Brill and Sandberg, 1967; Myer, 1968a), or various aromatic chromophores (Goodman *et al.*, 1968), or a combination of both, could easily explain the observed differences.

The variations of the dichroic features in the Soret and the δ-absorption regions (300–370 nm, reflecting predominantly conformational alteration in the heme environment), the changes in the dichroic peaks ascribed to contributions from transitions of aromatic chromophores (the 289-, 282- and possibly the 250-nm inflections; Myer, 1968b), and the 260-nm inflection (sensitive to the histidine–iron coordination linkage; Flatmark and Robinson, 1968) seem to fall into two distinct classes. The first class is associated with the changes occurring during the initial stages of NBS modification and reaching a limit in the case of these preparations at 3–4 equiv of NBS, and the second class is of alterations reflected by the preparations at higher than 3–4 equiv of the reagent. The dichroic changes of the first type are characterized by (i) the alteration of the complex Soret dichroic spectrum of the native protein to a partially simplified form, *i.e.*, containing both positive and negative Soret inflections at 404 and 418 nm, the former with increasing ellipticity and the latter with decreased rotatory strength; (ii) the lack of changes in the inflections at 289 and 282 nm; (iii) the replacement of the doubly inflected spectrum below 275 nm with a single band of lower rotatory strength; and (iv) a more or less unchanged dichroic pattern in the region 300–370 nm (Figure 2). The second type of dichroic variations are characterized by the replacement of the partially simplified Soret spectrum with a single symmetrical peak of decreasing rotatory strength, and the elimination of almost all dichroic features in the δ-absorption and the aromatic absorption regions of the spectrum. Structural alteration at higher than 3–4 equiv

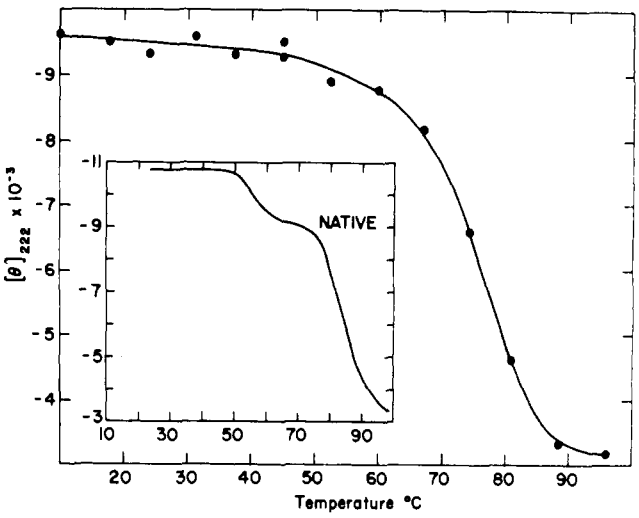

FIGURE 3: Effect of temperature on ellipticity at the 222-nm band for the native and 3:1 NBS-modified horse heart ferricytochromes *c*. pH 7.0 in water. Inset: data for the native protein.

of NBS, in general, results in drastic simplification of the entire visible spectrum, whereas alteration occurring up to about 3–4 equiv is merely the partial simplification of the Soret spectrum. Compared to the CD studies of the systematically perturbed native molecule by the denaturing agent, urea, or by increasing temperatures (Myer, 1968b), the first type of dichroic alterations, both in the visible and the intrinsic absorption regions, exhibit striking similarity to the changes observed during the first step of the denaturation process of the native molecule. The resulting dichroic spectrum at the last stage of the NBS reaction, however, is very similar to that observed for simple heme–peptide systems isolated from the native molecule with pepsin and/or trypsin hydrolyses, essentially devoid of any secondary structure as compared to the native protein (Myer and Harbury, 1966; MacDonald, 1972).

In contrast to the thermal denaturation of the native protein, the NBS-modified preparation at 3 equiv of NBS undergoes unfolding by a single-step transition centered at about 75°, which is about 8° lower than that of the major transition of the native molecule (Figure 3). In addition, the magnitude of the change of ellipticity resulting from thermal denaturation is of the same order as observed for the major denaturation step of the parent molecule (Myer, 1968b; Figure 2). The thermal denaturation profile of the 3:1 NBS-modified preparation therefore is similar to that of the native protein which has been partially unfolded during the first phase of the thermal and urea denaturation processes (Myer, 1968b). Thus the conformational alterations of the protein resulting from chemical modification during the early steps of the reaction are similar to those occurring in the native protein during the first phase of the denaturation processes. The denaturation of the native protein has been attributed to the elimination or uncoupling of electronic interactions, such as dipole–dipole interactions, between the prosthetic group and the protein moiety with apparently little or no change in either the polypeptide conformation of the molecule or the coordination configuration of the heme iron (Myer, 1968b).

The effects of pH variation on the Soret CD spectra for the 3:1 NBS-modified preparation and the native protein are compared in Figure 4A,B. As in the case of native ferricytochrome *c*, the Soret CD spectrum of the modified prep-

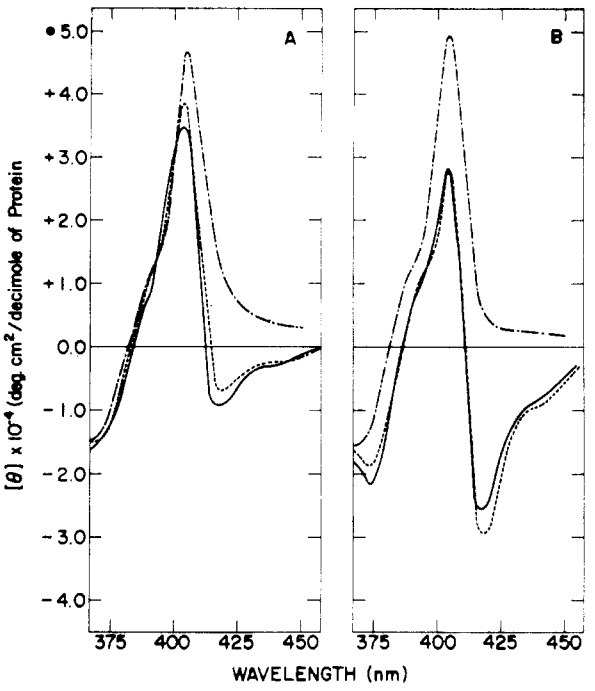

FIGURE 4: Effect of pH on the Soret CD spectra of the native and the 3:1 NBS-modified horse heart ferricytochromes *c*. (A) 3:1 NBS-modified preparation. (B) Native. ——, pH 7.0; - - - - - - -, pH 4.0; —·—·—, pH 10.0.

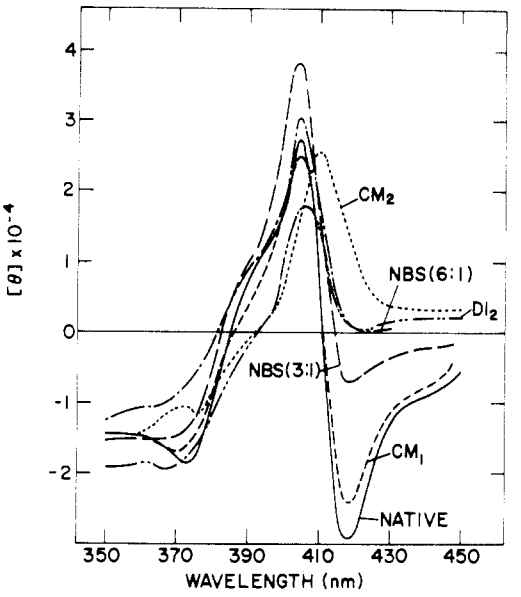

FIGURE 5: Comparison of Soret spectra of native, NBS-modified, and other chemically modified preparations of horse heart ferricytochromes *c*. Conditions: pH 7.0; 0.1 M phosphate buffer; 22°. ——, native; — — —, 3:1 NBS-modified preparation; —·—·—, 6:1 NBS-modified preparation; - - - - - -, CM_1 (monocarboxymethylated derivative; ·····, CM_2 (dicarboxymethylated derivative); —·—·—·—, DI_2 diiodo derivative.

aration is more or less insensitive to pH variation between 4 and 7, although there are distinct differences in the dichroic spectra of the two molecules. The change of pH to alkaline values results in simplification of the complex Soret spectra in both cases, and the resulting spectra are almost indistinguishable from one another. Therefore it seems that although small but significant conformational alterations in the molecule have indeed occurred upon initial NBS modification, the proton-linked conformational functions of the modified preparations, up to 3–4 equiv of NBS, are more or less indistinguishable from those of the native protein.

The Soret dichroic spectrum of the 3:1 NBS-modified preparation, representative of the terminal stage of the first step of the reaction, and of the 6:1 NBS-modified preparation, representative of the middle of the second step, are compared with those for the monocarboxymethylated, dicarboxymethylated, and the diiodo derivatives of the protein in Figure 5. The monocarboxymethylated derivative (CM_1) containing a single carboxymethylated methionine, residue 65, and a single carboxymethylated histidine, residue 33 (Harbury, 1966; Ando *et al.*, 1965), exhibits a dichroic spectrum which is very similar to that observed for the native protein except for a small decrease in ellipticity at the 418-nm inflection. The additional carboxymethylation of the second methionyl residue, methionine-80 (CM_2), however, produces a dichroic spectrum which differs from that of the native protein as well as the 3:1 NBS-modified preparation in terms of both the complexity and the location of the peak (Figure 5). Similarly, the Soret dichroic spectrum of the diiodo derivative (DI_2, containing substitutions of tyrosyl residues at positions 65 and 74; McGowan and Stellwagen, 1970) is different from those of both the native protein and the 3:1 NBS-modified preparation (Figure 5). A similar situation concerning the Soret CD spectrum of the 3:1 NBS-modified preparation is also evident from comparison with the results of the native protein in the presence of extrinsic ligands (Myer, 1968b). Thus it seems that as far as the conformational degradation of the molecule in terms of the electronic and chemical configuration of the central-coordinated complex is concerned, the 3:1 NBS-modified preparation is clearly discernable from the other systems mentioned above. The dichroic spectrum of the NBS-modified preparation representative of the terminal stages of the reaction, the 9:1 preparation, is however very similar to that of the diiodo derivative (Figures 1 and 5); also the simplified form of the Soret band suggests gross identity to CM_2 and the native protein in the presence of extrinsic ligands.

Discussion

The spectroscopic variation upon incremental additions of NBS to ferricytochrome *c*, the chemical characterization of the products at various stages of the reaction, and the alteration of the two aspects of the biological function lead one to conclude that the NBS modification of the protein can be best interpreted in terms of the formation of two consecutive reactions (see Myer, 1972). The formation of the first reaches a maximal value at about 3.5 equiv of NBS and represents the first spectroscopic step. It contains modification of tryptophan-59, methionine-65, and possibly tyrosine-74, and it exhibits continuous derangement of the reducibility property of the protein, but the oxidizability function is more or less unchanged. The second of the forms first appears at about 2 equiv of the reagent, and it reaches a maximum at the limit of the reaction investigated, *i.e.*, 9 equiv of the reagent. The formation of the second form is reflected in the second spectroscopic step, and structurally it represents the oxidation of the second methionyl residue, residue 80, concurrent to which there is a loss of the oxidizability function of the protein as well. The elucidation of the conformational implications of the chemical alterations in these two molecular

forms is the subject of this article, and therefore, the discussion is limited primarily to this aspect of the problem.

Consideration of the nature of the differences between the circular dichroism spectra of NBS-modified preparations in the order of increasing modification indicates that these differences can be adequately interpreted in terms of the sequential formation of at least two conformationally distinct molecular forms of the protein. Since the demarcation point between the two classes of dichroic changes is the same as that observed for the spectral distinction between the first and the second steps (Figure 2 of Myer, 1972), the two modified molecular forms are the same as the two conformationally distinct forms implied from these investigations. The dichroic spectrum of the 3:1 NBS-modified preparation is characteristic of the first form, and the second form is dichroically represented by the 9:1 preparation. The two molecular forms produced sequentially upon addition of NBS are therefore not only structurally and spectroscopically distinct (see Myer and Pal, 1972), but are also conformationally distinct from each other and from the native protein.

The CD spectrum of the first molecular form maintains many of the dichroic characteristics of the native protein. The ellipticity at 222 nm, representing the magnitude of organized polypeptide structures (Holzwarth and Doty, 1965; Schellman and Schellman, 1964), is more or less unaltered; the ellipticity of the aromatic bands at 282 and 289 nm are unchanged; the general details of the spectrum in the δ-absorption region vary only slightly, if at all; and the complexity of the Soret CD band—*i.e.*, the doubly inflected spectrum—is preserved (Figures 1 and 2). Thus in terms of the gross conformation of the protein and the aromatic chromophores contributing to the 282- and the 289-nm bands, the first NBS molecular form is more or less indistinguishable from the native protein. The differences in the ellipticities of the Soret bands, both the negative and the positive limbs, in comparison to those observed for the native protein, and the replacement of the doubly inflected spectrum in the region 250–270 nm, which contains contributions from transitions sensitive to the nature of the central-coordinated complex (Flatmark and Robinson, 1968) (Figure 2), on the other hand, clearly show that the structural alteration in the first molecular form causes small, but significant, conformational changes in the protein. Since the differences are primarily in those spectral regions which reflect contributions from transitions associated with the heme moiety or its central-coordinated complex (Myer, 1968b; Flatmark and Robinson, 1968), the conformational alterations must be primarily of the prosthetic group or its immediate vicinity.

The dichroic spectrum of the first NBS molecular form (3:1 NBS-modified preparation, Figures 1 and 2) is very similar to that of the native protein at slightly elevated temperatures (refer to Myer, 1968b, Figure 1, curve C). Likewise, there are other similarities if one compares the differences in the dichroic spectra of preparations with increasing modification of the protein up to the 3:1-equivalent preparation (Figures 1 and 2) to those spectra observed during the first phase of thermal and urea denaturation of the native protein. These striking similarities indicate that the nature of the conformational changes resulting from chemical modifications in the first NBS molecular form are much like those in the native protein during the first phase of the thermal and the urea denaturation steps. In the native protein these changes have been ascribed to the uncoupling of the heme–protein interactions only with no alteration of either the nature of the central-coordinated complex or of the polypeptide moiety of the protein (Myer, 1968b). Therefore, a similar situation must also be the case in the first NBS molecular form.

If the above interpretation is valid, then, consistent with the observations in the case of the native protein (Myer, 1968b), it is to be expected that the denaturation profile of the 3:1 NBS-modified preparation (the preparation at the final stage of the first step of the reaction; see Myer, 1972) should be devoid of the first denaturation step. In addition, if forces such as dipole–dipole interactions, which add to the stabilization forces in the molecule, are indeed eliminated in the first NBS-modified molecular form, then its melting temperature should also be lower than that of the native protein. As shown in Figure 3, the results confirm both the above stipulations. Thus the apparent conformational change in the molecule resulting from chemical modification of the various functional groups during the first step of the reaction is the elimination or decrease of electronic interaction between the prosthetic group and the protein moiety. Concerning the effects of chemical modification on the nature of the central-coordinated complex in the 3:1 NBS-modified preparation, the alteration, if any, is definitely not of the type in which the intrinsic ligand is replaced by extrinsic ligand or by another protein ligand. Examination of dichroic spectra of altered coordination structure will demonstrate this point. The dichroic spectrum of the 3:1 NBS-modified preparation differs from that of the native protein in the presence of extrinsic ligands (Myer, 1968b, Figure 11) or 9 M urea (Myer, 1968b), in which the apparent change is the replacement of intrinsic coordinating group with extrinsic molecule, and the said spectrum differs from CD spectra of derivatives such as the diiodo and the dicarboxymethylated derivatives, in which the coordination configuration of the metal atom is altered (Harbury, 1966; Stellwagen, 1968; McGowan and Stellwagen, 1970). Possibly, as a consequence of conformational alteration, there is indeed a weakening of the central coordination bonds. This has been confirmed in the following article (Myer and Pal, 1972).

Thus the first molecular form of the NBS reaction is indeed conformationally deranged, but the alterations are rather localized in a small region of the protein and involve mainly the perturbation of the electronic interactions of the heme group. These perturbations do result in a slight weakening of the coordination linkage of the heme iron, but the molecule maintains the same coordination configuration of the central heme conplex. Consistent with earlier conclusions (see Myer, 1972), and with the information available from X-ray diffraction studies (Dickerson *et al.*, 1971), of the various structures modified in this form, the conformational variations seem to be associated with the tryptophan-59 residue only. The elimination of methionine-65 as the functional group is further supported by the observation that, although modification of this group does cause small variation in the Soret spectrum of the protein (curve for CM_1 *vs.* native in Figure 5), the variations are in no way as significant and as pronounced as when tryptophan-59 is also modified. Whether the electronic configuration of the heme is determined by direct participation of the indole moiety of tryptophan-59 (*i.e.*, through direct electronic interaction) or whether tryptophan maintains proper folding of the protein surrounding the heme group through hydrogen bonding with one of the heme side chains could not be determined from these investigations. It seems very likely that both mechanisms may be operational in establishing the role of this functional group in the protein.

The dichroic changes in the intrinsic absorption region, reflecting in the main the alterations of the organized struc-

tures of the protein (Schellman and Schellman, 1964) at higher than 3 equiv of the reagent, and the nature of the resulting dichroic curve for the preparation at the highest NBS/protein ratios, are characteristic of systems undergoing derangement of the polypeptide conformation of the molecule. The elimination of the dichroic peaks in the aromatic absorption region, the 282- and the 289-nm bands, the transformation of the doubly inflected Soret band to a single symmetrical positive peak, the replacement of the doubly peaked spectrum in the region 250–260 nm, and the elimination of the details in the δ-absorption region are typical of this protein when subjected to denaturation at high temperatures (Myer, 1968b). Thus the chemical modification during the second NBS step, or the formation of form II, results in not only the derangement of the secondary structures of the polypeptide chain, but also of the aromatic chromophores, and the prosthetic group, including possibly the chemical nature of the central-coordinated complex. The occurrence of gross conformational degradation during the second step of the reaction is not surprising, since the significant modification of the molecule from 3 to 9 equiv of NBS is the oxidation of methionyl residue 80 to the corresponding methionine sulfoxide (see Myer, 1972). In the native molecule methionine-80 provides the the sixth coordinating group to the heme iron, the configurational integrity of which has been shown to stabilize the polypeptide structures of the molecule and the electronic configuration of the heme moiety (Myer 1968b). The existence of altered heme conformation and changes in the chemical nature of the heme iron complex is further indicated from the observation that the Soret dichroic spectrum of the 9:1 NBS-modified form (Figure 2) is similar to that of the diiodo derivative (Figure 5); it has been shown in the diiodo derivative that definite changes occur in the central-coordinated complex (McGowan and Stellwagen, 1970). The differences between the Soret dichroic spectrum of the 9:1 NBS-modified preparaand those of the dicarboxymethylated derivative (Figure 5) or the native molecule in the presence of extrinsic ligand (Myer, 1968b), on the other hand, suggest that the changes in the central-coordinated complex in the 9:1 NBS-modified preparation must be different from those in the other two cases. The dicarboxymethylation results in modification of the methionine-80 sulfur to the *S*-carboxymethyl derivative, the addition of extrinsic ligand results in merely the replacement of methionine sulfur, while the product of the NBS reaction is methionine sulfoxide (Myer, 1972). The differences in the chemical nature of the central iron complex in form II of NBS-modified cytochromes could be due to structural alterations other than those of the methionyl residue or to differences in the nature of the substituents at methionine sulfur itself, *i.e.*, oxygen in the case of the NBS-modified preparation and the carboxymethyl group in the case of the CM_2 derivative, or a combination of the two. In view of the recently reported observation that methionine sulfoxide results in low-spin hemochromes in the simple heme–peptide systems, whereas the carboxymethylsulfonium salt fails to show coordination to heme iron (O'Brien, 1969), it seems the observed dichroic differences could be due to differences in the substituent groups in the two modifications. It has been shown that the formation of the dicarboxymethyl derivative does not alter the structural environment of the buried tyrosyl residues or of the tryptophanyl residue (Stellwagen, 1968). The NBS modifications at higher than 3 equiv, in addition to containing increasing amounts of oxidized methionine-80, contain altered structure of the tryptophanyl residue, methionyl residue 65, and at least one of the four tyrosyl residues. These notable differences and the above considerations indicate that the significance of additional altered structures in the molecule in determining its conformation cannot be ignored.

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