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Nitrocytochrome *c*. II. Spectroscopic Properties and Chemical Reactivity*

Abel Schejter, Irit Aviram, and Mordechai Sokolovsky

ABSTRACT: The oxidized form of cytochrome *c* nitrated at its tyrosyl-67 residue exists in four different spectral types in the pH range 2–10. In acid medium nitrocytochrome *c* is a pure high-spin compound, while in alkaline medium it is present in the low-spin state. At intermediate pH's, two forms are present, one with high-spin and the other with low-spin characteristics.

The effect of ascorbate upon the different forms and the pH dependence of the oxidized spectrum can be best interpreted by assuming that cytochrome *c* consists of two species with identical primary structure that differ in the position of the nitro group on the tyrosyl-67 residue, and that this residue is unable to rotate. One of these species is similar to native cytochrome *c* in its reducibility with ascorbate, but it shows a pK = 5.9 instead of the pK = 9 of the native enzyme; the other species is not reducible by ascorbate and remains in the high-spin state up to pH 8.6. Both forms are autoxidizable and bind carbon monoxide.

In studies on the effects of chemical modifications of amino acid residues of proteins, two types of phenomena are usually investigated: one is the reactivity of particular residues toward different reagents, that may be interpreted in purely structural terms: the other is the biological activity of the modified protein, whether enzymic, antigenic, or of some other kind. These aspects of the reaction of cytochrome *c* with tetranitromethane were considered in the accompanying paper (Sokolovsky *et al.*, 1970).

In the case of modifications of cytochrome *c*, additional points deserve investigation. This is because, by being a heme protein, cytochrome *c* is endowed with a wealth of spectroscopic, magnetometric, and chemical reactivity properties, the study of which provides a deeper insight into the relationships between the heme group and the protein moiety. This paper deals with such a study on nitrocytochrome *c* (Sokolovsky *et al.*, 1970).

Materials and Methods

Nitrocytochrome *c* was prepared as described in the preceding paper (Sokolovsky *et al.*, 1970).

Spectrophotometric titrations were performed on a Cary 14 spectrophotometer using equimolar solutions of nitrocytochrome *c* in 0.05 M glycine-HCl, acetate, phosphate, and Tris-

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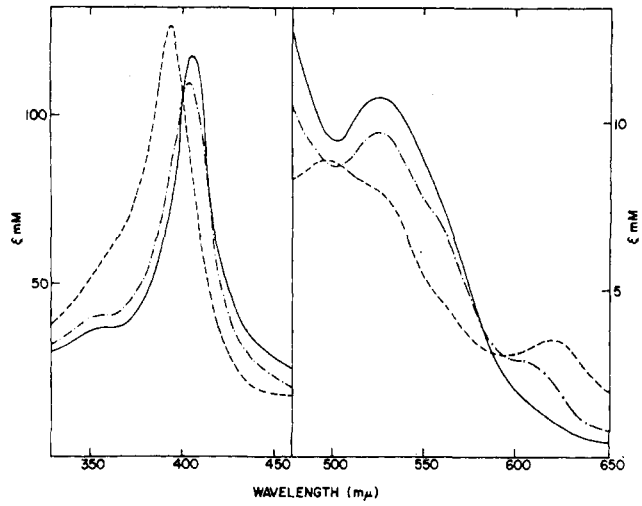

FIGURE 1: Visible spectrum of ferric nitrocytochrome *c* at various pH's: (– – –) pH 2, (–·–·–) pH 7, and (——) pH 10.

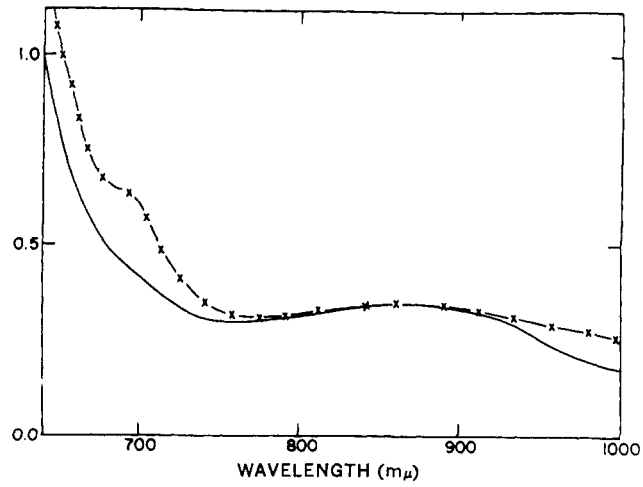

FIGURE 2: Near-infrared spectrum of ferric nitrocytochrome *c*. (X–X) pH 4 and (——) pH 7.

HCl buffers. The pH's were determined with a glass electrode in a Radiometer pH meter 25.

The reduction with ascorbate at different pH's was studied using Thunberg cuvets. In experiments at alkaline pH, ascorbate was added to nitrocytochrome *c* solution in Na_2CO_3–$NaHCO_3$ buffer (pH 10). The side arm contained an appropriate amount of HCl needed to lower the pH of the solution to any desired pH value. The tube was evacuated and the absorbance measured before and after HCl addition. For reduction at neutrality, HCl was added to the alkaline buffer before evacuation. The final pH was determined after the tubes were opened to air.

Titration with imidazole was performed by adding aliquots of a 0.02 M imidazole solution at pH 7.0 to nitrocytochrome *c* at the same pH. The spectra were recorded and from the readings at 610 mμ, corrected for the dilution, the stability constant of the complex was calculated.

Results

Spectral Properties of Nitrocytochrome c. Four spectroscopic types can be distinguished in ferric nitrocytochrome *c* solutions between pH's 2.0 and 10.0. The positions of the maxima of their spectral bands are listed in Table I, and the spectra are shown in Figures 1 and 2.

Type I, the form observable in strongly acid solutions, is similar to native ferricytochrome *c* in the same conditions. The peaks at 495, 620, and 900 mμ are characteristic of high-spin heme proteins (George *et al.*, 1961; Day *et al.*, 1967).

The transition from type I to type II has a spectroscopically operable p*K* of 2.5 (Figure 3A). Type II has rather peculiar spectral characteristics (Figures 1 and 2). It possesses a band at 528 mμ, typical of low-spin ferricytochrome *c*, together with bands at 605 and 870 mμ, characteristic of high-spin heme proteins. Furthermore, type II has a band at 695 mμ, observed only in native cytochrome *c* between pH's 2.5 and 9.0 (Theorell and Åkesson, 1941).

An ionization with p*K* = 5.9 separates type II from type III (Figure 3B). The two forms are spectroscopically very similar, except for the fact that the 695-mμ band is almost entirely absent from type III.

Finally, type III changes into type IV with a p*K* of 8.5 (Figure 3C). Type IV has a strong band at 530 mμ, no high-spin bands, and its Soret band is shifted from 405 mμ in types II and III to 407 mμ. These data indicate that type IV is a low-spin heme protein.

Oxidation–Reduction Properties of Nitrocytochrome c. The usual procedure of reduction with dithionite cannot be applied to nitrocytochrome *c* without affecting the nitro group, since dithionite is known to convert nitrotyrosyl into aminotyrosyl residues (Sokolovsky *et al.*, 1967). Types II and III can be partly reduced with ascorbate in aerobic conditions; the extent of this reduction differs slightly with the preparations observed, but in all cases it reaches values of 20–30% of

TABLE I: Positions of Soret, Visible, and Near-Infrared Absorption Bands of Ferric Nitrocytochrome *c* at Different pH's.

Type	I	II	III	IV
pH	2	4	7	>10
Absorption maxima (mμ)	900, 620, (565), (530) 495, 395	870, 695, 605, (565), (530) 528, 405	870, 605, (565), 528, 405	(565), 530 407

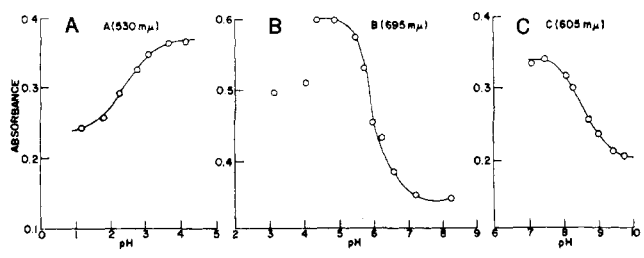

FIGURE 3: Spectrophotometric titration of ferric nitrocytochrome *c* in various pH ranges: (A) pH 1–4 (type I–type II); (B) pH 4–8 (type II–type III); (C) pH 7–10 (type III–type IV).

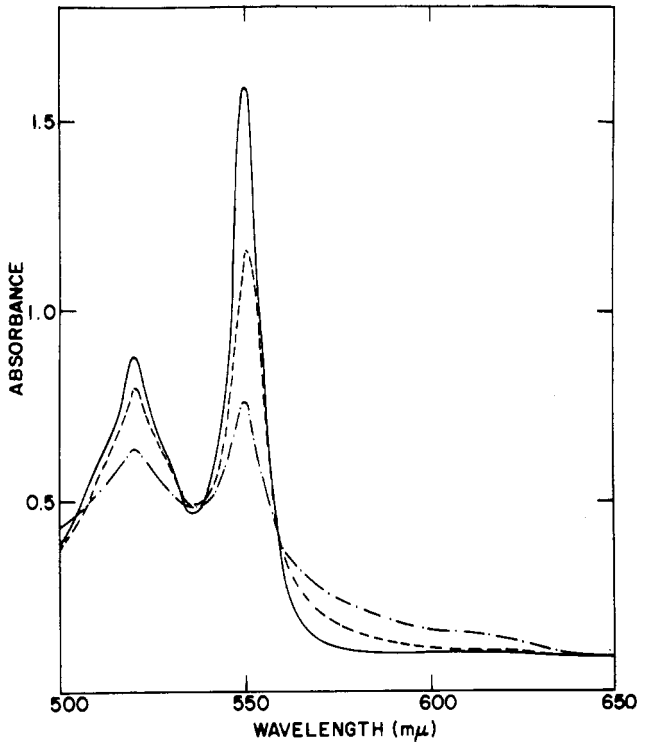

FIGURE 4: The visible spectra of nitrocytochrome *c* reduced by ascorbate. (——) Reduction performed at pH 10 in the absence of of air, (- - -) result of bringing the pH down to 7, and (-·-·-·) reduction performed at pH 7 in the absence of air.

reduction, if the extent of reduction is estimated by comparison with an equimolar solution of native cytochrome *c*. If the air is evacuated from the cuvets containing these partly reduced samples of types II and III, the extent of reduction increases, as shown by an intensification of the α and β bands. That this is not due to completion of the reduction is evidenced by the persistence in the spectrum of a band at 605 mμ, typical of the oxidized state of types II and III (Figure 4).

Type IV can be reduced with ascorbate at pH 10, yielding a spectrum essentially similar to that of native ferrocytochrome *c* (Figure 4). If this reduced species is brought to pH 6 or to pH 4 in the absence of oxygen, the spectrum changes rapidly: the absorption bands at 550 and 520 mμ decrease to about 70% of their original intensities. However, this does not imply reoxidation, since no bands appear concomitantly in the 600-mμ region. Finally, if under these conditions the solution is exposed to oxygen, the spectrum changes again, by a further decrease of the reduced bands to about 20–30% of reduction, with the reappearance of the 605-mμ band.

Reaction with Ligands. In neutral solution, ferric nitrocytochrome *c* forms a complex with cyanide that is spectroscopically similar to that obtained from the native enzyme. Upon addition of imidazole to neutral ferric nitrocytochrome *c*, a low-spin spectrum is obtained, as evidenced by the disappearance of the 605- and 870-mμ bands. The association constant for this complex at pH 7.0, estimated for the unprotonated imidazole species is 2.6×10^4 M^{-1}; this value for the native enzyme is 30 M^{-1} (Schejter and Aviram, 1969). The reduced form of nitrocytochrome *c*, either at pH 10.0 or at pH 6.0, binds carbon monoxide.

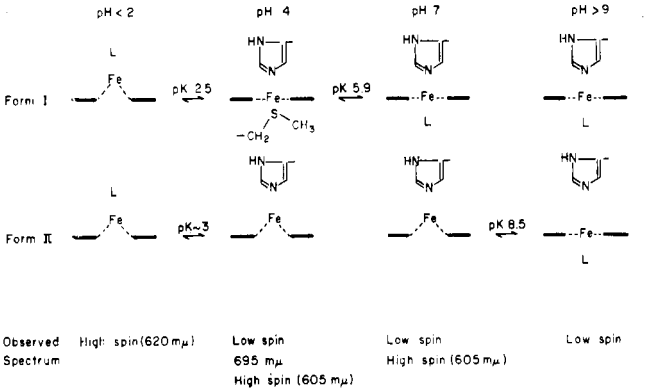

FIGURE 5: A scheme showing the proposed structures of the crevice of forms I and II of nitrocytochrome *c* at various pH's. The bold type lines represent the porphyrin plane; the dashed lines represent the porphyrin–iron bonds. The symbols above and below the Fe atoms represent the proposed protein ligands, where L stands for an unknown ligand.

Discussion

Since the empirical classification of heme protein spectra by Theorell (1942), it is known that the different spectral types are associated with different electronic configurations in the d orbitals of the iron. Crystallographic proofs have accumulated that show a direct relationship between the spin state of the iron and its position relative to the porphyrin ring (Hoard *et al.*, 1967; Glick *et al.*, 1967; Countryman *et al.*, 1969); in the low-spin state the iron lies in the plane of the porphyrin, while in the high-spin state it is displaced out of the plane. This is, furthermore, a consequence of molecular orbital theory (Zerner *et al.*, 1966). Recent nuclear magnetic resonance experiments on metalloporphyrins in solution suggest that when the latter are bound to two axial ligands the iron is coplanar with the porphyrin, while in cases where there is binding to only one ligand the iron is out of the porphyrin plane (Storm, 1970). These ideas will be the basis of our discussion of the different spectral types of nitrocytochrome *c*.

There is an apparent contradiction between the effect of nitration on the primary structure of cytochrome *c*, and the corresponding effect on the physicochemical properties of the molecule. On the one hand, primary structure studies indicate that tyrosyl-67 is the residue nitrated under defined experimental conditions (Sokolovsky *et al.*, 1970); on the other hand, both the spectrum of nitrocytochrome *c* and its oxidation–reduction reactions are best interpreted by assuming the presence of two different forms of the molecule.

The spectrum of the oxidized form is that of a high-spin heme protein below pH 2, and that of a low-spin protein above pH 10. In the intermediate pH region two species coexist, one with high-spin and the other with low-spin spectral characteristics. Such mixtures have already been observed in other heme protein derivatives, such as ferric myoglobin hydroxide (George *et al.*, 1961). However, these mixtures are equilibria between rapidly interconvertible isomers that differ only in the electronic configuration of their iron d orbitals; this appears not to be the case of nitrocytochrome *c*. In the first place, the spectrum in the pH region 2.5–5.9 shows both high-spin and low-spin bands, and a band at 695 mμ. The 695-mμ band has been assigned to a porphyrin to iron charge-

transfer transition (Eaton and Hochstrasser, 1967) theoretically predicted for low-spin ferric heme proteins (Zerner *et al.*, 1966), and, from empirical considerations, it has been correlated to the binding of the cytochrome *c* iron by methionyl sulfur (Shechter and Saludjian, 1967). Above pH 5.9, the 695-mμ band disappears, but the high-spin 605- and 870-mμ bands persist. If the form present between pH's 2.5 and 5.9 were a case of high-spin–low-spin equilibrium similar to that of ferric myoglobin hydroxide, upon raising the pH the whole equilibrium should be shifted toward a low-spin form lacking the 695-mμ band. That this is not so, as evidenced by the persistence of the high-spin bands, indicates that the two forms present between pH's 2.5 and 5.9 are not the two isomers of a rapidly attained equilibrium.

When nitrocytochrome *c* reacts with ascorbate in aerobic conditions at pH 4–7, it is only partly reduced. The species responsible for this reduction is present in an amount of 20% similar to that of the oxidized species responsible for the residual 695-mμ absorption that remains above the pK = 5.9 and disappears only above pH 10. Moreover, the residual enzymic activity of nitrocytochrome *c* is also 20% (Sokolovsky *et al.*, 1970). We attribute this behavior to a cytochrome *c* species nitrated at positions 48 or 74, that contaminates the major fraction, nitrated at position 67. The fraction that remains oxidized still shows low- and high-spin bands. When the air is evacuated from the solution, an increase in the partial reduction is observed, but the high-spin bands still remain. Again this indicates that there are two distinct molecular species present, one that possesses low-spin characteristics and is reduced by ascorbate, and another with high-spin configuration that is not reduced by the same reagent; if the two forms were in rapid equilibrium reduction of one would necessarily result in shifting the equilibrium until complete reduction was attained.

If the assumption that tyrosyl-67 nitrocytochrome *c* consists of two different molecular species is made, the pH dependence of the spectrum described above admits the following interpretation. Both forms, labeled I and II, exist in strongly acid solution in the high-spin configuration; the iron atom is not coplanar with the porphyrin ring. Form I undergoes an ionization with pK = 2.5 accompanied by a conformation change that causes the methionine-80 sulfur to coordinate the iron, while the latter becomes coplanar with the porphyrin and attains the low-spin state. The ionization with pK = 5.9 is a property of form I. This ionization is most probably the counterpart of the ionization observed in native ferricytochrome *c* with pK = 9.0. The latter has been analyzed kinetically, and interpreted as involving two different processes: a fast unprotonation, with a pK of 11.0, and a slow conformation change with an equilibrium constant of about 100 (Schejter *et al.*, 1969; Schejter, 1970). The nature of the group ionizing with pK = 11 is unknown. However, a pK of 11 has been assigned to a tyrosyl residue of ferric cytochrome *c* (Rupley, 1964). If this is the nitrated residue, its pK should be lowered by about 3 pH units, as it happens in the nitration of phenol. The fact that the pK of form I is 5.9 is entirely consistent with this assignment, provided that the change in conformation that accompanies this ionization is of the same kind and degree as that operating in native cytochrome *c* with pK = 9.0. Finally, form II undergoes an ionization with pK = 8.5 that results in its conversion into a low-spin form.

With respect to the experiments of reduction with ascorbate, the interpretation is a follows. Form I can be reduced with ascorbate either below or above its pK = 5.9; form II becomes reducible only above its pK = 8.6. When both forms are reduced at pH 10, and brought to neutral or weakly acid pH in the absence of air, the observed decrease in the intensities of the visible bands suggest the appearance of a species in the reduced high-spin state. It is noteworthy that reduced dicarboxymethylmethionyl (65,80)–cytochrome *c* has a very similar pH dependence of its spectrum: the α and β bands are weaker in neutral solution, and reach their full intensity only at pH 10 (Schejter and Aviram, 1970). Finally, it is also necessary to emphasize that both forms of nitrocytochrome *c*, I and II, are rapidly oxidized by air, even in the presence of an excess of ascorbate. From the correlation between spectral properties and reducibility with ascorbate, it seems a necessary condition for the latter property that the iron be in its low-spin state. This result is in keeping with the fact that the reduction of native cytochrome *c* with ascorbate is not impaired by the formation of a complex with imidazole (Schejter and Aviram, 1969). The π system of the porphyrin has been shown to constitute a very plausible pathway for electron transfer to the iron, both by theoretical considerations (George and Griffith, 1959) and by experimental results (Castro and Davis, 1969). Furthermore, in the case of cytochrome *c* one edge of the porphyrin ring is exposed to the solvent, while other ways of access to the iron are sterically impeded (Dickerson *et al.*, 1967). Hence, a possible reason for the correlation between low-spin state and ease of reducibility with ascorbate may be found in the coplanarity of the iron with the porphyrin that attends the low-spin state. Under these conditions, there is maximal overlap between the iron $d_{x^2y^2}$ orbital and the π orbitals of the porphyrin, which should facilitate the porphyrin-mediated electron transfer.

This condition of coplanarity seems necessary, but not sufficient, since the low-spin form of dicarboxymethylmethionyl–cytochrome *c* that exists in neutral solutions is not readily reducible by ascorbate (Schejter and Aviram, 1970), and the same applies to the cyanide complex of ferric cytochrome *c* (Tsou, 1952). It is important to point out that both forms of reduced nitrocytochrome *c* bind carbon monoxide, while in the oxidized state the reaction with imidazole is much more favorable than in the case of native cytochrome *c*. The increased reactivity implies that upon nitration there is a significant loosening of the closed crevice structure (Margoliash and Schejter, 1966; George *et al.*, 1967). This, together with the fact that the binding of the methionine sulfur is restricted to a narrow pH range in type I and completely impeded in type II, shows that the tyrosyl-67 residue affects the iron binding properties of the methionyl-80 sulfur, and that these two residues are close to each other in the native molecule.

The question that remains to be answered is that of the difference between the two forms of nitrocytochrome *c*. This is apparently incompatible with the structural studies that show that tyrosyl-67 is the residue nitrated in the major fraction of nitrocytochrome *c* (Sokolovsky *et al.*, 1970). A plausible solution for this paradox is the following. We assume that tyrosyl-67 is partly buried, so that access of the reagent to one of the two ortho positions is easier than to the other. Hence, the rates of nitration of the two ortho positions will be different, and the molar ratio of the products will be the same as the ratio of the rates of their formation. Furthermore, we assume that the nitrotyrosyl phenyl rings are sterically

hindered from rotation around their bonds to the tyrosyl β-carbon. Under such circumstances, the two forms will not be equivalent in their physicochemical properties, and will not be rapidly interconvertible, but this difference will not be detectable by primary structure studies. Of course, it is also possible to assume that the rotation of the phenyl rings is sterically hindered already in the native protein; the result will be the same. Work is now in progress in order to assess the reliability of this hypothesis.

Added in Proof

Dr. R. E. Dickerson has informed us that this interpretation is in the keeping with the position and environment of tyrosine-67 in his 2.8-Å resolution model of cytochrome *c*.

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Thiolysis of Dinitrophenylimidazoles and Its Use during Synthesis of Histidine Peptides*

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ABSTRACT: The thiolytic cleavage of 2,4-dinitrophenylimidazoles was applied to the synthesis of histidine-containing peptides.

This paper describes the preparation and properties of starting materials for such syntheses and illustrates their use in the synthesis of some peptides containing histidine at either the C or the N terminus. A spectrophotometric method for following the extent of thiolysis of 2,4-dinitrophenylimidazoles was developed and used to establish optimal reaction conditions for the quantitative removal of the protecting group from histidine residues in aqueous and nonaqueous media.

Synthesis of histidine peptides can be performed without protection of the imidazole ring (Holley and Sondheimer, 1954; Hofmann *et al.*, 1957). However, the basicity of imidazole may trigger undesirable side reactions and lead to low yields of coupling and problems of purification (Schröder and Lübke, 1965). Commonly used protecting groups for the imidazole ring are the benzyl group (du Vigneaud and Behrens, 1937; Theodoropoulos and Gazopoulos, 1960) or the carbobenzoxy group (Patchornik *et al.*, 1957; Akabori *et al.*, 1958; Shaltiel and Patchornik, 1963). The benzyl group is sometimes inadequate, since its removal by catalytic hydrogenolysis is a slow process which often does not reach completion (Kopple *et al.*, 1963), while the removal by means of sodium in liquid NH_3 is a drastic procedure which may even cause rupture of peptide bonds (Guttmann, 1963). Protection

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