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Nitrocytochrome *c*. I. Structure and Enzymic Properties*

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ABSTRACT: Cytochrome *c* was nitrated with tetranitromethane at pH 8.0.

A modified enzyme nitrated specifically at the tyrosyl residue in position 67 was purified. This nitrocytochrome *c* was found to be unable to restore the respiratory function of cytochrome *c* depleted mitochondria. In the presence of cyanide, which forms a complex with cytochrome *c*, the described nitration did not take place.

Specific chemical modifications of amino acid side chains are often used to establish whether particular amino acid residues in a protein are required for its biological activity. Experimentally, tyrosine is one of the most accessible residues of proteins (Vallee and Riordan, 1969). Horse heart cytochrome *c* contains four tyrosyl residues. Their ionizations were studied by spectropolarimetry (Ulmer, 1966, and references cited therein), and their reactivities by acetylation reactions. The present communication reports the preparation using tetranitromethane, and the enzymic activity of nitrotyrosyl-67-cytochrome *c*. The accompanying paper describes the physicochemical properties of this modified enzyme (Schejter *et al.*, 1970). A preliminary report has been given (Schejter and Sokolovsky, 1969) and similar studies were reported also by Skov *et al.* (1969).

Materials

A crystalline preparation of cytochrome *c* from horse heart was a gift of Dr. E. Margoliash. Commercial horse heart cytochrome *c*, type II, was obtained from the Sigma Chemical Co., and purified on Amberlite CG-50 (Margoliash and Walasek, 1967). Tetranitromethane was obtained from Fluka AG; α-chymotrypsin, three-times crystallized, from Worthington; and Dowex AG 50-X2 (200–400 mesh) was obtained from Bio-Rad. All other chemicals were of the best grade available.

Methods

Concentrations of native cytochrome *c* were determined by the absorbance of the reduced form at 550 mμ using a molar absorptivity of 2.77×10^4 M^{-1} cm^{-1} (Margoliash and Frohwirth, 1959). The concentration of nitrated cytochrome *c* was determined by amino acid analysis.

A Zeiss PMQ spectrophotometer was used for measurements of absorbance at single wavelengths, and Cary 15 or 14 recording spectrophotometers were employed for determination of absorption spectra.

The pH of solutions was measured on a Radiometer Model 26 pH meter equipped with a glass-calomel combination electrode.

Amino acid analysis were performed with a Beckman-Unichrome amino acid analyzer according to the procedure of Spackman *et al.* (1958). Samples were hydrolyzed in constant-boiling HCl in evacuated sealed tubes in the presence of 10 μl of phenol, at 110° for 22 hr. Tryptophan was determined on the unhydrolyzed protein using *N*-bromosuccinimide (Spande and Witkop, 1967), by the reaction with dimethylaminobenzaldehyde (Spies and Chambers, 1949), and after acid hydrolysis in the presence of 2% thioglycolic acid (Matsubara and Sasaki, 1969). Methionine was also determined after alkaline hydrolysis (Neumann, 1967). Quantitative determinations of nitrotyrosine were obtained from amino acid analyses as described by Sokolovsky *et al.* (1966).

Paper chromatography was carried out on Whatman No. 3MM paper using 1-butanol–pyridine–acetic acid–water (60:40:12:48, v/v) as the solvent.

Cytochrome c activity was estimated by the method of Jacobs and Sanadi (1960).

Nitration was performed by addition of tetranitromethane in 95% ethanol to a solution of ferricytochrome *c* (2–2.5 mg/ml) in 0.1 M Tris–0.1 M KCl (pH 8.0) at room temperature. The final concentration of ethanol was always less than 4%. The reaction was terminated by passing the mixture through a Bio-Gel P-4 column in 0.04 M ammonium bicarbonate (pH 8.0). In experiments using cyanide, 0.1 M KCN was substituted for 0.1 M KCl.

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TABLE I: Nitration of Cytochrome *c* with Increasing Molar Excesses of Tetranitromethane: Nitrotyrosine Content.[a]

Moles of Tetranitromethane/Mole of Cytochrome	Nitrotyrosyl Content (Moles/Mole of Cytochrome)
2	0.06
4	0.13
8	0.20
12	0.30
24	0.68
42	1.2
60	1.3

[a] Nitration was carried out in 0.1 M Tris–0.1 M KCl (pH 8.0) for 35 min at room temperature.

TABLE II: Time Course of Nitration[a] of Cytochrome *c* with Tetranitromethane; Nitrotyrosyl Content[b] and Absorbance at 695 mμ.

Time (min)	Nitrotyrosyl Content, NO_2Tyr/Mole	% Changes of *A* at 695 mμ[d]
0	0	0
5	0.24	20
10	0.50	47
15	0.74	70
20	0.90	>90
35	1.2	>95
35[c]	0.2	0
45	1.3	>95
45[c]	0.3	0

[a] Nitration was carried out with a 40-fold molar excess of tetranitromethane, 0.1 M Tris–0.1 M KCl (pH 8.0). [b] Determined by amino acid analyses. [c] Nitration was carried out with a 40-fold molar excess of tetranitromethane, 0.1 M Tris–0.1 M KCN (pH 8.0). [d] Considering as 100% the change observed for preparation containing 1.5 moles of nitrotyrosyl residues/mole of cytochrome *c*.

Chymotryptic Digest and Peptide Map. A chymotryptic digest of 100 mg of nitrocytochrome *c* was obtained by incubating it with 11.5 mg of chymrotypsin in 0.1 M ammonium bicarbonate (pH 8.0) for 10 hr at room temperature. The final volume was 5 ml. Peptide mapping of the chymotryptic digests was performed with 0.15 μmole of digested nitrocytochrome *c* on Whatman No. 3MM paper. The samples were subjected to electrophoresis in pyridine-acetate (pH 6.4) for 90 min at 50 V/cm, followed by chromatography for 14 hr. The peptides were located by dipping in 0.5% ninhydrin in ethyl alcohol. Peptides containing nitrotyrosine were detected by the yellow color which appears when the peptide maps are exposed to ammonia vapors.

Fractionation of Peptides. The solution of the chymotryptic hydrolysate was chromatographed on G-50 Sephadex (medium, 1.8 × 180 cm) at room temperature with the use of 0.04 M ammonium bicarbonate (pH 8.0). The yellow fraction was pooled and lyophilized. The pooled fraction containing 8 μmoles of nitrotyrosyl peptides was dissolved in 1 ml of 0.2 M pyridine-acetate buffer (pH 3.1), and chromatographed on a column of Dowex AG 50-X2 resin (200–400 mesh, 1 × 95 cm) at 45° using an elution gradient of pyridine-acetate at pH 3.1–5.0. A flow rate of 20–25 ml/hr was maintained and 2-ml fractions were collected. The absorbances of the effluent fractions were measured at 280 and 360 mμ. The fractions were pooled, lyophilized, and their nitrotyrosine content was determined after acid hydrolysis. Samples of 0.2 μmole of fractions 1–4 were further purified by paper chromatography while fractions 5–7 were purified by electrophoresis followed by paper chromatography. Nitrotyrosine peptides were eluted with 0.01 M ammonium bicarbonate from the yellow areas which were cut out before ninhydrin staining.

Results

The course of nitration of cytochrome *c* was followed with different molar excesses of tetranitromethane at pH 8.0 for 35 min at room temperature (Table I). The degree of nitration depends on the molar excess of tetranitromethane; thus, with 8-fold molar excess of tetranitromethane only 0.2 tyrosyl residue was nitrated while with 42 molar excess of tetranitromethane 1.2 nitrotyrosyl residues were formed. The degree of nitration with 8-fold molar excess of tetranitromethane could be increased if the reaction mixture was constantly stirred. However, under these conditions there was some destruction of tyrosine, namely, the sum of nitrotyrosine plus tyrosine was lower than expected. Therefore stirring was avoided and the time course of nitration of cytochrome *c* was followed with a 40-fold molar excess of tetranitromethane at pH 8.0, in the presence and absence of cyanide (Table II). Within 35 min, 1.2 nitrotyrosyl residues were introduced, with concommitant changes in the absorbance at 695 mμ. These changes were prevented by the presence of cyanide and from the degree of nitration (Table II) it seemed as if one especially reactive tyrosine residue which is essential for the band at 695 mμ was protected from nitration by the presence of the ligand. The same results could be obtained by using an 8-fold molar excess of tetranitromethane, but a longer period of reaction (4 hr without constant stirring) was necessary. In order to minimize side reactions (Sokolovsky *et al.*, 1970), the use of 40-fold molar excess of tetranitromethane for 30–35 min was adopted for routine preparation.

Nitrocytochrome *c* could be resolved into three components by chromatography on Amberlite CG-50 (Schejter and Sokolovsky, 1969). Amino acid analysis (Table III) revealed that the fraction bound most tightly to the column, fraction III was unreacted cytochrome *c*, while fraction I (8–10% yield), contained more than 2 nitrotyrosyl residues. Fraction II, the major component, contained 1.05 moles of nitrotyrosine/mole of protein.

Increasing the concentration of cyrochrome *c* in the nitration mixture from 2 to 5–6 mg per ml does not change the rate of nitration, but causes an increase in the yield of fraction I (*ca.* 30%). For large-scale preparation of nitrocytochrome *c*, 200–300 mg, the nitrated enzyme eluted from the column of

TABLE III: Amino Acid Composition of Ferricytochrome *c*[a] Nitrated with a 40-fold Molar Excess of Tetranitromethane, and of Fractions Obtained by Chromatography on Amberlite.[b]

Amino Acid	Native Cyto-chrome *c*	Nitrated Cyto-chrome *c*	Frac-tion I	Fraction II	Frac-tion III
Lys	19.3	19.1	19.4	19.3	19.2
His	3.2	3.0	3.1	3.1	3.2
Arg	2.2	2.2	2.3	2.1	2.2
Asp	8.4	8.2	7.8	8.3	8.1
Thr	9.5	9.8	9.9	9.1	9.5
Glu	12.6	12.6	11.8	12.4	12.4
Pro	4.1	3.7	3.2	3.9	4.1
Gly	11.8	11.8	11.5	12.1	11.9
Ala	6	6	6	6	6
Val	2.9	2.9	3.1	3.0	2.9
Met	1.75	1.65	1.3	1.8	1.5
Ile	5.8	5.8	5.4	5.9	5.9
Leu	5.9	6.0	5.6	5.9	5.8
Tyr	3.6	2.2	0.9	2.5	3.4
Phe	3.9	4.1	3.7	3.9	3.9
NO_2-Tyr		1.2	2.3	1.05	
Trp[c]	0.90	0.81		0.79	

[a] Results expressed as moles of amino acid per mole of enzyme and calculated on the basis of 6 alanines/mole. [b] Nitrated cytochrome *c* was adsorbed onto 1 × 10 cm column of Amberlite CG-50 (Na^+) and eluted with a linear Na^+ concentration (Schejter and Sokolovsky, 1969). [c] See Methods.

Bio-Gel was diluted with an equal volume of 0.02 M sodium phosphate buffer (pH 7.8) adsorbed on the ion-exchange column and the latter was washed with 500 ml of this buffer. Nitrocytochrome *c* (fraction II) was eluted with 0.17 M NaCl in the phosphate buffer (pH 7.8). The first bulk of enzyme which emerged from the column contained about 1 mole of nitrotyrosine/mole of protein. The other fractions eluting after this modified protein contained less than 0.6 mole of nitrotyrosine/mole of protein, and were discarded.

Peptide maps of chymotryptic digests of cytochrome *c* nitrated in the absence of cyanide have 6 yellow peptides appearing upon exposure to ammonia and an additional spot, peptide A, which is yellow even before exposure to NH_3 (Figure 1). The peptides were eluted and all, except peptide A, were found by amino acid analysis to contain nitrotyrosine. Peptide C which was composed of glutamic acid and nitrotyrosine at a ratio of 1:1 did not appear in the peptide map of the enzyme nitrated in the presence of cyanide. The other peptides were not pure enough and because of the very low yield of isolation after further purification of these peptides, preparative isolation by ion-exchange chromatography was preferred.

The chymotryptic digest of nitrocytochrome was chromatographed on Sephadex G-50 (1.8 × 180 cm). Two main colored fractions were resolved. The fast fraction was red and contained the heme peptide and only 6% of the total nitrotyrosine while the slow fraction was yellow and contained

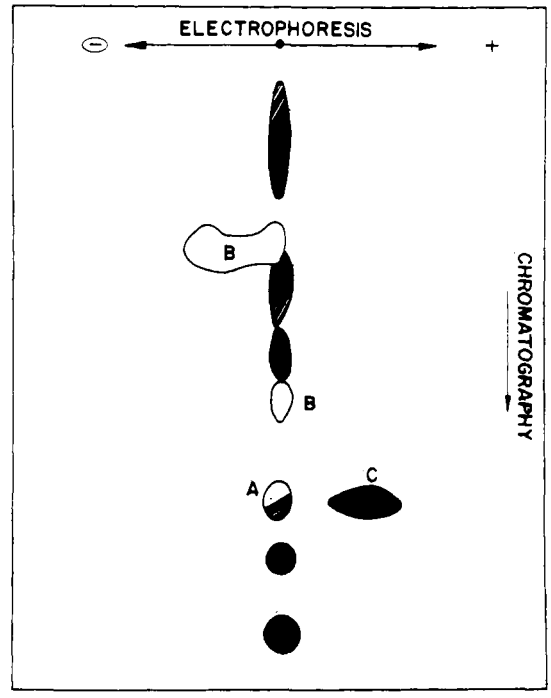

FIGURE 1: Peptide map showing the position of the yellow peptides of chymotryptic digest of nitrocytochrome *c* after exposure to NH_3. Peptide C is the one which does not show up in the peptide map of the enzyme nitrated in the presence of cyanide. Peptide A was yellow before treatment with NH_3 and peptides B are the heme peptide derivatives.

85% of the total nitrotyrosine which was applied to the column. The yellow fraction was chromatographed on Dowex AG 50-X2. Figure 2 shows the elution pattern of peptides and indicates the portions of the eluate that were combined into fractions. No other yellow peptides were eluted even when the column was washed with NH_4OH up to pH 11.5. Only

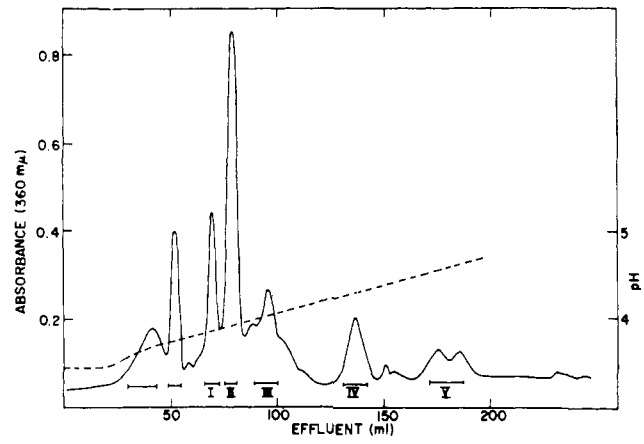

FIGURE 2: Elution diagram of chymotryptic hydrolysate of nitrocytochrome *c*, obtained by chromatography on Dowex AG 50-X2 (1 × 95 cm) at 45°. Peptides were eluted with a gradient of pyridine-acetate at pH 3.1–5.0. Detection of nitrotyrosyl peptide was determined by the absorbances of the effluent fractions at 360 mμ. The dashed line represents the pH of the eluate and the bars on the abscissa mark the fractions pooled.

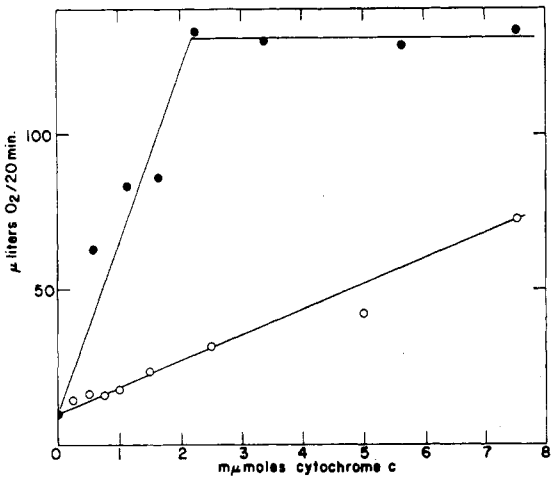

FIGURE 3: Respiratory activity of cytochrome *c* depleted rat liver mitochondria in the presence of exogenous native horse heart cytochrome *c* (●–●) and nitrotyrosyl (67) cytochrome *c* (○–○). The oxygen consumption was measured in a Warburg respirometer, in a reaction mixture containing 9 mg of mitochondrial protein; the composition of the medium was as given by Jacobs and Sanadi (1960).

those fractions which contain more than 5% of the total nitrotyrosine which was applied to the ion-exchange column were further purified by chromatography. The yellow peptides were eluted and the results of amino acid analyses of acid hydrolysate of these purified peptides are shown in Table IV. On the basis of these analyses all the yellow peptides can readily be located within the amino acid sequence of horse heart cytochrome *c* (Margoliash, 1962); peptide Ia, Glu-Tyr-Leu, position 66–68; peptide Ib and II; Glu-Tyr, position 66–67; peptide IIIa, Lys-Glu-Glu-Thr-Leu-Met-Glu-Tyr-(Leu), position 60–68; peptide IIIb, Thr-Tyr, position 47–48; peptide IV, Gly-Asp-Val-Glu-Lys-Lys-Ile-Phe, position 1–10; and peptide V, Leu-Glu-Asp-Pro-Lys-Lys-Tyr, position 68–74.

Enzymic Activity. It was previously reported (Schejter and Sokolovsky, 1969) that the addition of nitrocytochrome *c* to cytochrome *c* depleted mitochondria restored the respiratory function of the latter. This, however, requires relatively large concentrations of the modified enzyme. A typical experiment is shown in Figure 3, where the rate of oxygen consumption of depleted mitochondria is drawn as a function of the concentration of exogenous cytochrome *c* or nitrocytochrome *c*. The maximal respiration rate is achieved when the concentration of the exogenous native enzyme in the system is about 2 mμmoles/9 mg of mitochondrial protein. For an equimolar concentration of nitrocytochrome *c*, only 16% of the maximal respiration is obtained. In order to reach maximal respiration rate, the exogenous nitrocytochrome *c* must be at least 13 mμmoles/9 mg of mitochondrial protein.

Discussion

The reaction of horse heart ferricytochrome *c* with a 40-fold molar excess of tetranitromethane for 35 min and at pH 8.0, yields, as the major product, a modified cytochrome in which one of its four tyrosyl residues has been nitrated, while its spectral and functional properties have been altered. Under

TABLE IV: Amino Acid Composition of Yellow Peptides of Chymotryptic Digests of Nitro Cytochrome *c*.[a]

	Peptide						
	Ia	Ib	II	IIIa	IIIb	IV	V
Amino Acid	Residues/Molecule of Peptide						
Lysine				1.0[b]		3.1	1.9
Histidine							
Arginine							
Aspartic acid						1.2	1.1
Threonine				1.1	1.0[b]		
Serine							
Glutamic acid	1.1	1.1	1.0[b]	2.8		1.4	1.1
Proline							1.0
Glycine						2	0.3
Alanine							
Valine						1.0[b]	
Methionine				0.9			
Isoleucine						0.9	
Leucine	1.0[b]			1.3			1.0[b]
Tyrosine				0.6			0.1
Phenylalanine						1.0	
Nitrotyrosine	1.0	1.0[b]	1.0	0.4	0.8		0.8
Yield (%)	9	4.5	42	7	6	5	8.5

[a] Results are from selected single analyses. If each fraction contained more than one yellow peptide after chromatography they were designated a and b in the table. [b] Arbitrarily selected as 1 mole for reference.

these conditions of nitration, tetranitromethane does not react only with tyrosyl and cysteinyl residues, but might react also with tryptophanyl and methionyl residues as well (Sokolovsky *et al.*, 1969, 1970). Since the single tryptophanyl residue present in cytochrome *c* might have been highly reactive, chemical analysis were performed to check this possibility. More than eight-tenths of an intact tryptophanyl residue were detected by the NBS method (Spande and Witkop, 1967), by the method of Spies and Chambers (1949), and by amino acid analysis of acid hydrolysates performed in the presence of 2% thioglycolic acid (Matsubara and Sasaki, 1969).

The possibility that methionine residues were oxidized by the reaction with tetranitromethane was ruled out since neither methionine sulfone, after alkaline hydrolysis (Neumann, 1967) nor methionine sulfoxide were detected.

Modification of the vinyl side chains of the myoglobin porphyrin ring by tetranitromethane were recently described by Atassi (1969). However, in cytochrome *c*, this reaction cannot occur since these reactive side chains are saturated and bonded to the two cysteinyl residues.

Thus, it seems likely that under the conditions described above cytochrome *c* was selectively modified at a tyrosyl residue, and if any other alterations did occur they were of minor consequences. It should be noted that the sum of nitrotyrosine plus unreacted tyrosines after the nitration was usually close to the value of tyrosine obtained from native enzyme. However, when the reaction mixture of the enzyme and tetranitro-

methane was stirred constantly, or when commercial preparations of enzyme not highly purified were used, destruction of up to one tyrosyl residue was occasionally observed.

Chymotryptic digests followed by peptide separation and analysis show that the major component of the mononitrocytochrome *c* is a species in which the tyrosine residue at position 67 has been nitrated. Two other tyrosines were nitrated at a lower degree, *i.e.*, less than 10% yield each, and they are at positions 48 and 74. It should be noted that the ion-exchange chromatography could resolve species which contain on the average 1 mole of nitrotyrosine from those that have either more than 2 moles or less than 0.5 mole of nitrotyrosine per mole of protein. Hence, the mononitrocytochrome *c* might be composed of 70–80% molecules which were nitrated at tyrosine-67 and 20% which were nitrated at 48 or 74. If we accept the above assumption the activities measured could be explained on this basis. If the loss of activity is due to the nitration at tyrosine-67 then, since there are about 20% of the species which are nitrated at any other position which in turn do no affect the biological activity, we might expect a residual activity of about 20% of that of the native cytochrome *c*. In addition, as shown in the accompanying report (Schejter *et al.*, 1970) the band at 695 mμ of nitrocytochrome *c* at pH 7.0 is not completely abolished. Since the band at 695 mμ is correlated usually with the biological function of cytochrome *c* this will indicate that the residual activity is due to a species similar to native cytochrome *c* and not to a new species which has different biological properties resulting in reduced efficiency in electron transport.

A puzzling problem is the presence of a peptide both in the peptide map (Figure 1) and in the ion-exchange chromatography of the chymotryptic peptides (Table IV), which does not contain any nitrotyrosyl residue. From the peptide map, because of the low yield, it was difficult to determine its position in the primary structure of the protein though we feel it might be a partially modified tryptophanyl residue. Amino acid analysis of the protein revealed the loss of a small fraction of tryptophan which will support the assumption that this unidentified peptide contains a partially modified tryptophanyl residue. However, the amino acid composition of the peptide isolated from the Dowex column seems to fit the N-terminal end of the protein. Since the N-terminal end does not contain any of the amino acid side chains susceptible to tetranitromethane, the most reasonable explanation for this finding is that nitroformate ion is associated with this peptide, which results in the appearance of a yellow spot even before exposure to ammonia. Work now in progress should offer the explanation for this modified peptide, and for the fact that the peptide isolated from the map is not similar to the one isolated by ion-exchange chromatography.

Preferential nitration of tyrosyl residues in proteins is thought to be due to an unusual microenvironment which lowers the apparent dissociation constant of the phenolic hydroxyl group (Riordan *et al.*, 1967a) since it is the ionized form of tyrosine which reacts with tetranitromethane (Sokolovsky *et al.*, 1966). While the state of ionization of tyrosyl residues in cytochrome *c* is still unclear, there is at least one residue which has a p*K* lower than the rest (Rupley, 1964). Alternatively, or in addition, nitration of the other tyrosyl residues might be retarded because they are "buried" in the interior of the protein. Iodination of *Candida krusei* cytochrome *c* modifies the tyrosyl residues at positions 73 and 80 (Narita *et al.*, 1968) which correspond to positions 67 and 74 in the mammalian cytochrome *c*. This implicates tyrosine-67 as a highly reactive residue. It has been shown that in guanidylated cytochrome *c* which was acetylated with acetylimidazole, only one tyrosyl residue appears to be responsible for functional alterations (Cronin and Harbury, 1965). Ulmer (1966) showed that in horse heart ferricytochrome *c* two tyrosines were acetylated with acetylimidazole and therefore suggested that in this protein there are two exposed and two buried tyrosyl residues. It should be noted that nitration of only one tyrosyl residue of ferricytochrome *c* which differs from acetylation and iodination is very similar to the observations made on carboxypeptidase A (Riordan *et al.*, 1967b), in which nitration modifies one tyrosyl residue, while acetylation and iodination modify two tyrosyl residues. The fact that a species containing two nitrotyrosyl residues could be isolated is in keeping with the assumption that there are two "free" tyrosyl, one of which is kinetically more reactive. Since the yields of nitration of tyrosyl-48 and -74 are very similar, it is difficult at this stage to conclude whether tyrosines-67 and -48, or -67 and -74, are the two "free" residues. A number of successive chemical modifications is now being undertaken in order to settle this question.

The high reactivity of tyrosyl-67 compared to that of -48 or -74 can be also rationalized by assuming that tyrosyl-67 is in a "basic" environment. Thus, the rate of nitration of copolymers of tyrosine and other amino acids progressively decreases in the following order: Lys-Tyr (10:1) > Glu-Lys-Tyr (54:40:6) > Glu-Tyr (9:1) (Sokolovsky *et al.*, 1966).

That the microenvironment of tyrosyl-67 is an essential factor of its reactivity with tetranitromethane is borne out by the drastic curtailment of this reactivity when the reaction is performed on cytochrome *c* in the presence of cyanide. Formation of the cyanide complex results in the displacement of methionyl-80 from its iron binding position (Schejter and Aviram, 1970), as well as in changes in the conformation of the protein (George *et al.*, 1967). It is most probable that this change in conformation affects principally the residues closest to methionyl-80. Hence, these results tend to suggest that between tyrosyl-67 and methionyl-80 exists a close relationship in native cytochrome *c*, disrupted or modified by the binding of cyanide.

Another explanation stems from the fact that the tyrosyl-67 residue is part of the environment of the crevice, as it can be inferred from crystallographic studies at-4Å resolution (Dickerson *et al.*, 1967). Therefore, disruption of the crevice could be expected to alter the chemical properties of the tyrosyl-67 residue, or of its hydrophobic microenvironment, rendering it unreactive toward tetranitromethane.

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

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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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