NITRATION OF HORSE HEART FERRICYTOCHROME C WITH TETRANITROMETHANE

Abel SCHEJTER and Mordechai SOKOLOVSKY
Department of Biochemistry, Tel-Aviv University, Tel-Aviv, Israel

Received 5 July 1969

1. Introduction

Specific chemical modification of amino acid side chains helps to establish whether a particular amino acid residue in a protein is required for biological activity. In studies on the structure and function of cytochrome *c*, we have employed tetranitromethane (TNM) which has been shown to be a convenient reagent for the nitration of tyrosyl residues in proteins [1]. In this communication, we describe the nitration of a single tyrosyl residue of ferricytochrome *c* with TNM at pH 8.0. The product is fully active but displays an altered spectrum. The 695 mμ absorption band at pH 7.2 disappeared and a new band appeared at 590–620 mμ. The spectral shifts could be interpreted, taking into account the change in ionization constant resulting from nitration of the tyrosyl residue.

2. Materials and methods

Horse heart cytochrome *c*, type II, was obtained from the Sigma Chemical Co., and purified on Amberlite CG-50 [2]. Tetranitromethane was obtained from Fluka AG.

Nitration was performed by addition of TNM in 95% ethanol to a solution of ferricytochrome *c* (2.5 mg/ml) in 0.1 M Tris – 0.1 M KCl, pH 8.0, at room temperature. The reaction was terminated by passing the mixture through a Bio-Gel P-4 column. The degree of nitration was determined by amino acid analysis [1].

Amino acid analyses were carried out with a Beckman-Unichrome amino acid analyzer by the technique of Spackman et al. [3]. Tryptophan was determined on the unhydrolyzed protein using N-bromosuccinimide [4].

Cytochrome *c* activity was estimated by the method of Jacobs and Sanadi [5].

3. Results

The time course of the nitration of cytochrome *c* was followed with a 40-fold molar excess of TNM at pH 8.0 (table 1). Within 30 min, 1.2 nitrotyrosyl residues are introduced with a concomitant change in the

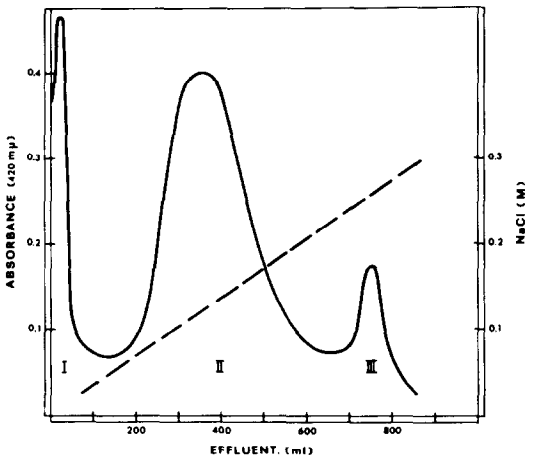

Fig. 1. Chromatography of nitrocytochrome *c*; 6 μmoles of nitrocytochrome were adsorbed onto a 1 × 10 cm column of Amberlite CG-50 (Na^+), and eluted with a linear Na^+ concentration. (Dilute solution 0.02 M sodium phosphate buffer pH 7.8.) The dashed line represents the NaCl concentration

Table 1
Time course of nitration of cytochrome *c* with TNM; nitrotyrosyl content, absorbance at 695 mμ and activity.

Time (min)	Nitrotyrosyl content NO_2-Tyr/mole	Percent changes of absorbance at 695 mμ	Enzymic activity mmO_2/mg/min
0	0	0	1.2
5	0.24	20	–
10	0.50	47	1.1
15	0.74	70	1.1
20	0.90	91	–
30	1.2	97	1.1
30[a]	0.2	0	1.05
45	1.4	100	1.1
45[a]	0.3	0	1.05

[a] Nitration was carried out with a 40-fold molar excess of TNM, 0.1 M Tris-0.1 M KCN, pH 8.0.

Table 2
Tyrosine and nitrotyrosine content of ferricytochrome *c* nitrated with a 40-fold molar excess of TNM, and fractions obtained by Amberlite chromatography.

Amino acid	Native cytochrome *c*	Nitrated cytochrome *c*	Fraction I	Fraction II	Fraction III
Tyr	3.6	2.2	0.9	2.5	3.4
NO_2-Tyr	–	1.2	2.3	1.05	–

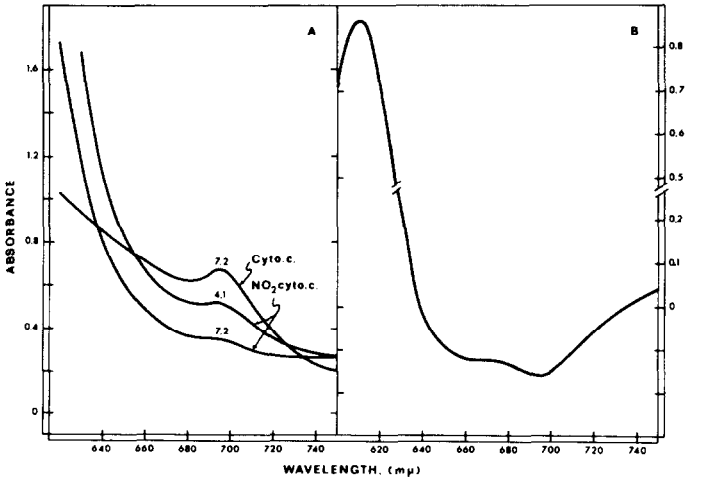

Fig. 2A. The absorption spectra of cytochrome *c* and nitrocytochrome *c*, at the pH values indicated, all at 8.4×10^{-4} M. B. Difference spectrum of nitrocytochrome *c* minus cytochrome *c* at 8×10^{-4} M 0.04 M phosphate (pH 7.2).

absorbance at 695 mμ. These changes are prevented by the presence of cyanide. The enzymic activity of nitrocytochrome *c* was tested by incorporating it into cytochrome *c* depleted mitochondria, and measuring respiration [5]. There is no alteration in activity even after 45 min of reaction (table 1). Restoration of the respiratory activity is complete and indistinguishable from the effect of native cytochrome *c*. For routine preparation a reaction time of 30 min was adopted, the time required for the maximum spectral change.

Nitrocytochrome *c* could be resolved into three components by chromatography on Amberlite CG-50 (fig. 1). Amino acid analysis (table 2) revealed that fraction III, bound most tightly to the column, was unreacted cytochrome *c*, while fraction I contained more than 2 nitrotyrosyl residues. Fraction II, the major component, was homogeneous by electrophoresis on acrylamide gel and contained 1.05 moles of nitrotyrosine per mole of protein.

Nitration of one tyrosyl residue causes the disappearance of the 695 mμ absorption band at pH 7.2 (fig. 2A). However, the spectrum of nitrocytochrome *c* is pH-dependent and at lower pH the 695 mμ band reappears. In addition a new absorption band in the region of 590–620 mμ with a maximum at 612 mμ at pH 7.2 is seen clearly in a difference spectrum of the nitrated versus the native cytochrome (fig. 2B).

Spectrophotometric titration of the 695 mμ band,

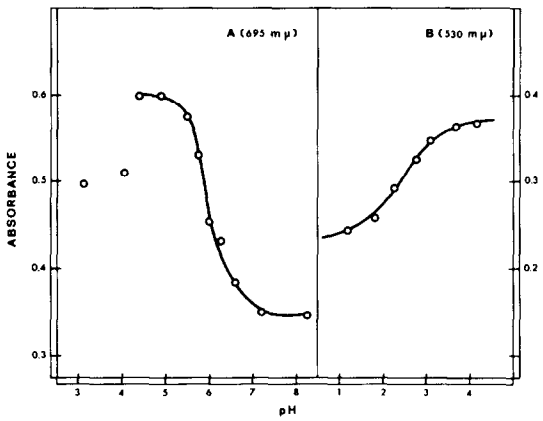

Fig. 3. Spectrophotometric titration of (A) 8.4×10^{-4} M nitrocytochrome *c* in 0.1 M phosphate-acetate buffer at 695 mμ. (B) 3.6×10^{-5} M nitrated cytochrome *c* in 0.1 M glycine-HCl or acetate buffers at 530 mμ.

due to the heme-linked ionization of nitrocytochrome *c*, gives an apparent pK of about 5.9 (fig. 3A). Furthermore, titration of nitrocytochrome *c* at 530 mμ (fig. 2B), reveals a heme-linked ionization with an apparent pK 2.5 similar to that of native cytochrome *c* [6].

Nitrocytochrome *c* cannot be reduced by ascorbate, cysteine and ferrocyanide. Reduction by dithionite ($Na_2S_2O_4$) alters the spectrum to resemble that of native, reduced cytochrome *c*. However, dithionite also rapidly reduces the nitrotyrosyl to an aminotyrosyl residue [7]. In fact, nitrotyrosyl reduction appears to proceed even more rapidly than the conversion of the iron from the ferric to the ferrous form.

4. Discussion

Preferential nitration of tyrosyl residues in proteins has been thought to be due to an unusual microenvironment which lowers the apparent dissociation constant of the phenolic hydroxyl group [8] since it is the ionized form of tyrosine which reacts with TNM [1]. While the state of ionization of tyrosyl residues in cytochrome *c* is still unclear, it would appear that there is at least one residue which has a pK lower than the rest [9]. Alternatively, or in addition, nitration of the other tyrosyl residues might be retarded because they are "buried" in the interior of the protein. The fact that a species containing two nitrotyrosyl residues could be isolated is consistent with the findings of Ulmer that two tyrosines could be acetylated with acetyl imidazole [10].

It cannot be stated as yet if nitrocytochrome *c* is a distinct species or if different tyrosines are partially nitrated. Its chromatographic behavior suggests the former and preliminary peptide isolation studies are consistent with this hypothesis. No evidence for an interaction of TNM with residues other than tyrosine has been found so far. Comparison of the peptide patterns of the modified cytochrome with that of the native protein (work now in progress) should offer final proof that neither methionine nor tryptophan have been attacked. The heme peptide derived from cytochrome *c* by chymotryptic digestion reacts slowly with TNM and therefore it will be necessary to

rule out that the heme itself is not attacked by TNM in the intact protein.

Since nitration of tyrosine increases its acidity by about 3 pH units [8] a similar shift in the pH dependence of cytochrome *c* heme-linked ionization might be expected upon nitration of the protein. This indeed appears to be represented by the shift of the 695 mμ band heme-linked ionization from a pK of about 9 in the native enzyme [6], to an apparent pK of 5.9 in the nitrated enzyme (fig. 3A). This implication of a tyrosyl group as responsible for the alkaline ionization in the native enzyme is supported by studies of the pH dependence of the kinetics of this change [11,12]. The lack of reducibility of nitrocytochrome *c* is in keeping with this hypothesis, since the alkaline form of the native enzyme is also more resistant to reduction [13].

The presence of a band at 590–620 mμ in nitrocytochrome *c* can be explained in two different ways: either it is the 695 mμ band shifted to a higher energy, due to a movement of the methionine-80 sulfur ligand towards the iron, or it indicates the presence of a high spin component in the nitrated protein. The latter, however, seems to be inconsistent with the general low spin type of the rest of the visible spectrum.

Despite its lack of reducibility, nitrocytochrome *c* is able to function when incorporated into cytochrome *c* depleted mitochondria. Since the 695 mμ band is usually considered a sign of the structural integrity of the molecule this is indeed a striking result that requires further experiments in order to be explained.

Acknowledgement

The authors wish to thank Moshe Moldovan and Am Gabso for excellent technical assistance.

References

[1] M.Sokolovsky, J.F.Riordan and B.L.Vallee, Biochemistry 5 (1966) 3582.
[2] E.Margoliash and O.F.Walasek, in: Methods in Enzymology, eds. R.W.Estabrook and M.E.Pullman, Vol. 10 (Academic Press, New York, 1967) p. 342.
[3] D.H.Spackman, W.H.Stein and S.Moore, Anal. Chem. 30 (1958) 1190.
[4] T.E.Spande and B.Witkop, in: Methods in Enzymology, ed. C.H.W.Hirs, Vol. 11 (Academic Press, New York, 1967) p. 498.
[5] E.E.Jacobs and D.R.Sanadi, J. Biol. Chem. 235 (1960) 531.
[6] E.Margoliash and A.Schejter, Advanc. Protein. Chem. 21 (1966) 113.
[7] M.Sokolovsky, J.F.Riordan and B.L.Vallee, Biochem. Biophys. Res. Commun. 27 (1967) 20.
[8] J.F.Riordan, M.Sokolovsky and B.L.Vallee, Biochemistry 6 (1967) 358.
[9] J.A.Rupley, Biochemistry 3 (1964) 1648.
[10] D.D.Ulmer, Biochemistry 5 (1966) 1886.
[11] A.Schejter, L.Davis and G.P.Hess, Fed. Proc. 28 (1969) 894.
[12] A.Schejter, Proc. Fourth Colloquium of the Johnson Research Foundation, Univ. of Penn., Philadelphia (1969) in press.
[13] K.G.Brandt, P.C.Parks, G.H.Czerlinski and G.P.Hess, J. Biol. Chem. 241 (1966) 4180.