Biochimica et Biophysica Acta, 527 (1978) 309—318

BBA 68616

IMPLICATION OF A TYROSYL RESIDUE AT THE ACTIVE SITE OF MITOCHONDRIAL L-MALATE:NAD$^+$ OXIDOREDUCTASE

HUGH B. OTWELL *, ALLEN YUNG-HO TAN and MICHAEL E. FRIEDMAN **
Chemistry Department, Auburn University, Auburn, Ala. 36830 (U.S.A.)
(Received May 2nd, 1978)

Summary

The tyrosyl residues of porcine mitochondrial L-malate:NAD$^+$ oxidoreductase (EC 1.1.1.37) have been studied spectrophotometrically and using selective chemical modification with iodine and tetranitromethane. CNBr hydrolysis and Sephadex G-25, G-50 and G-75 chromatography produced a peptide which contained two tyrosines in the native and the nitrated molecules when the nitration took place in an NAD$^+$-oxaloacetate solution. Nitration in the absence of the substrates caused the tyrosyl residues to disappear. Spectrophotometric titrations indicate that one of the 10 tyrosyl residues in mitochondrial L-malate: NAD$^+$ oxidoreductase titrate abnormally, while iodination experiments suggest that two fast-reacting tyrosines are not involved in activity. Nitration and iodination experiments, in conjunction with CNBr-mapping, suggest that two of the four nitrated tyrosyl residues are necessary for biological action. Titration of the sulfhydryl groups with 4,4-bis-dimethylaminodiphenyl carbinol before and after nitration indicate that none of the cysteinyl residues were oxidized by the tetranitromethane, thus ruling out the loss of enzyme activity due to the thiol oxidation.

Introduction

It has been suggested that the tyrosyl side chains of enzymes may be involved in either stabilizing the structure of proteins, by means of internal hydrogen bonds with side chains such as aspartic or glutamic acid, or the activity by means of the ionization of the phenolic hydroxyl group [1—3].

Several methods for determining the degree of tyrosyl residue exposure to the solvent have been employed [4,5]. Products from reaction with tetranitromethane [6] or iodine [7] have been used to locate these residues and deter-

* Postdoctoratal Research Associate, Department of Chemistry, University of South Carolina.
** To whom all inquires should be sent.

mine their importance towards enzyme activity.

Several amino acid residues have been implicated as essential in L-malate: NAD^+ oxidoreductase (EC 1.1.1.37) [8—10].

We have tried to establish the possible environments of the tyrosyl residues and then to locate the site in which an active tyrosine is found. These investigations also demonstrate that modification of the tyrosyl residues and not oxidation of sulfhydryl side chains leads to the loss of activity upon reaction of the enzyme with tetranitromethane.

Experimental section

Materials. Mitochondrial L-malate:NAD^+ oxidoreductase was either purified in this laboratory from pig heart according to the method of Wolfe and Nieland [11], or purchased from the Sigma Chemical Co. (Lot Nos. 40C-4710, 54C-95702 and 100C-953195). The Sigma enzyme was further purified as previously described [12]. Characterizations of both enzyme sources were made in the following manner: ultracentrifugation was performed in a Beckman-Spinco Model E ultracentrifuge at 59 640 rev./min using Schlieren optics and this resulted in a single peak. Polyacrylamide gel electrophoresis was carried out on a 7.5% acrylamide gel at pH 8.3 [13] and showed a single band for the laboratory preparation and a very faint second band for the commercial preparation. Inhibition by high concentrations of oxaloacetate (which is indicative of the mitochondrial form [14]) was observed for both sources. Spectrophotometric determination of the number of tyrosines [15] yielded 9.4 ± 0.5 tyrosines per mol enzyme. This result is comparable to the values of 9 and 10 as previously reported [9,16]. A kinetic study was made using oxaloacetate and saturating concentrations of NADH, and the double reciprocal plots yielded K_m values of 0.44 mM and 0.38 mM for the purchased and laboratory prepared enzymes, respectively (which are in good agreement with the value of 0.37 mM [17]).

NAD^+ (Lot No. 69B-630), CM-cellulose and Sephadex were purchased from the Sigma Chemical Co.; tetranitromethane and L-malic acid were obtained from the Aldrich Chemical Company. CNBr was purchased from Eastman Organic Chemicals. Dr. J.H. Harrison generously supplied us with a gift of 4,4-bis-dimethylaminodiphenyl carbinol; all other chemicals were of reagent grade unless otherwise indicated.

Reversible spectrophotometric titration. The titrations were carried out in a 0.15 M KCl solution. The pH was measured using a Beckman Zeromatic pH meter (Model No. 96) while the absorbance was read at 295 nm, using 1-cm quartz cells, in a Hitachi Spectrophotometer (Model No. 139). In each experiment, 2.5 ml 1—2 mg/ml protein solution was used. The pH was first increased by the addition of μl alequots of 1 M NaOH. 10-μl aliquots of 1 M HCl were then added to the protein solution after the pH remained constant from the NaOH addition.

Nitration. Nitration of the tyrosyl residues [6] was accomplished by adding aliquots of stock tetranitromethane reagent to solutions containing between 0.01 and 0.02 mM enzyme in a 0.05 M Tris-HCl buffer (pH 8.0 at 25°C). The stock reagent was prepared by diluting 50 μl tetranitromethane to 0.5 ml with 95% ethanol.

Nitration was also performed in the presence of NAD^+ and oxaloacetate (substrates for L-malate:NAD^+ oxidoreductase). Since oxaloacetate is an inhibitor of the enzyme at high concentrations, only 0.7 mM was used in the kinetic studies while 150 mM was used in the CNBr hydrolysis studies. A 3-fold molar excess of NAD^+, with respect to the enzyme, was used in all protection experiments. The enzyme was incubated at 25°C for 1 h with these substrates before nitration was performed. The enzyme was repeatedly assayed over a 2 h period, at the end of which the reaction was stopped by dilution with water. The nitrated derivative was separated from the nitroformate ion by gel filtration on Sephadex G-25 (which was first equilibrated with distilled water). The nitrated enzyme was collected, concentrated and then brought to 0.01 mM with distilled H_2O. The number of nitrated tyrosines were determined by measuring the absorbance at 381 nm using 2200 $M^{-1} \cdot cm^{-1}$ as the molar absorptivity.

Iodination. Iodination of the enzyme was performed in 0.05 M phosphate buffer (pH 7.0 at 4°C). Freshly prepared 9.0 mM I_2 in 0.04 M KI was added to a series of enzyme solutions such that succeeding enzyme solutions will contain increments of 2 mol I_2 per mol enzyme. The amount of enzyme in solution was between 0.45 and 4.50 μmol. The reaction was allowed to continue until the color disappeared, at which time it was assumed that the reaction was completed. The activity was recorded after the color disappeared. The rest of the samples were saved for spectral analysis.

Ultraviolet difference spectra. The ultraviolet difference spectra of the iodinated derivatives were obtained using a Perkin-Elmer double-beam recording spectrophotometer. The reference solutions were brought to pH 2.5 with HCl while the sample solutions were brought to pH 11.5 with NaOH. The higher pH was sufficient to cause the tyrosyl residues in all the iodinated derivatives to be ionized [18]. The absorbance was scanned from 350 to 250 nm.

Protein determinations. The analyses were made either by the method of Lowry et al. [19], or by using the molar absorptivity of 24 900 $cm^{-1} \cdot M^{-1}$ at 280 nm.

Amino acid analyses. Protein samples were hydrolyzed at 110°C with 6 M HCl for 20—24 h and the hydrolyzates were analyzed either on a Beckman model 121 M amino acid analyzer or a Beckman model 119 C amino acid analyzer equipped with an automatic integrator.

Enzyme assay. Enzyme assays were carried out according to previously defined specifications [20]. The assay solution contained 0.1 M glycine, 0.01 M L-malate and 0.2 mM NAD^+ (pH 9.5). The increase in absorbance of NADH at 340 nm was followed.

Sulfhydryl titration. Sulfhydryl groups were titrated with 4,4-bis-dimethylaminodiphenyl carbinol [21]. Approx. 1.7 mg of either nitrated or native enzyme were dialyzed against 0.04 M sodium acetate buffer (pH 5.1) 4 M guanidine hydrochloride. Several concentrations between 0.25 mM and 1 mM were used. 100 μl of each enzyme concentration was added to 5 ml aqueous solutions of 2.2 mM carbinol reagent. The reaction was completed in 30 min at room temperature. At the end of this time, the absorbance of the carbonium immonium ion was measured at 612 nm.

Cyanogen bromide digestion. CNBr hydrolysis of the enzyme was performed by a modification of the method of Gross and Witkop [22]. The enzyme was

dialyzed against distilled water and then lyophilyzed. It was then dissolved in 70% formic acid to give a final concentration of 10—20 mg/ml. CNBr was weighed directly into 5 ml 100% formic acid (due to its high volatility), and the concentration was made approx. 80 times greater than that of the enzyme. CNBr solution was then added to the enzyme and stirred for 48 h at 5°C. At the end of the reaction period, distilled water was added to dilute the formic acid to 10% and the sample was again lyophilyzed. These hydrolyses were performed on the following; the native enzyme, the enzyme which had been reacted with a 1460-fold excess of tetranitromethane and the enzyme reacted with a 1460-fold excess of tetranitromethane in the presence of NAD^+ and oxaloacetate.

The peptides produced by the CNBr hydrolyses were separated on 66 X 3.8 cm columns of Sephadex G-25, G-50 and G-75, which had been equilibrated with 30% acetic acid. The peptides were analyzed by polyacrylamide gel electrophoresis, descending paper chromatography (in *n*-butanol/acetic acid/pyridine/water, 15 : 10 : 3 : 12, v/v) and by high voltage electrophoresis (at 60 V/cm in pyridine/acetic acid/water, 10 : 100 : 2890, v/v).

Results

Spectrophotometric titrations were performed in order to determine the number of normal (exposed to the solvent) and abnormal (buried) tyrosyl side chains. The enzyme solution used in the spectrophotometric titration curve (Fig. 1) was initially brought to pH 6.0 and the absorbance read at 295 nm. The molar absorptivity (A_M) is plotted against the pH of the enzyme solution. The points for both the forward and reverse titrations fall on a smooth curve up until pH 12, signifying that the titration is reversible over that pH range. The absorbance remained constant after 1 min (suggesting that a slow unfolding of the protein was not taking place) for both the forward and backward titrations over the range pH 6—12. When the pH was brought above 12 there was a slow increase in the absorbance. This increase reached a maximum upon standing for 1 h. After the pH had been taken above pH 12, the titration could no longer be reversed, indicating an irreversible unfolding of the protein structure. The average pK of the reversibly titrated phenolic groups was found to be 10.4 ± 0.2, which is in agreement with values reported previously.

Spectrophotometric titration of the tyrosyl residues in model compounds should yield an A_M of 2260—2330 at 295 nm [23,24]. Using 2330 as the A_M for each tyrosyl residue and a calculated value of 18 200 (with a variation of ±700) for the ΔA_M of the enzyme between pH 9.5 and 11, 7.8 ± 0.3 tyrosyl residues were found to be reversibly titrated. The molar absorptivity change for the irreversible portion of the curve (broken line) was calculated to be 3000 ± 700 which gives a value of 1.3 ± 0.3 tyrosyl residues titrating abnormally. The total number of tyrosyl residues (9.1 ± 0.3) agrees favorably with the results of others [16].

The determination of reactive tyrosyl side chains was measured in the following experiments. The data from the nitration of the phenyl ring by tetranitromethane are given in Table I. Inhibition of the enzyme was not observed until the tetranitromethane reached a 146 M excess over the enzyme. At this

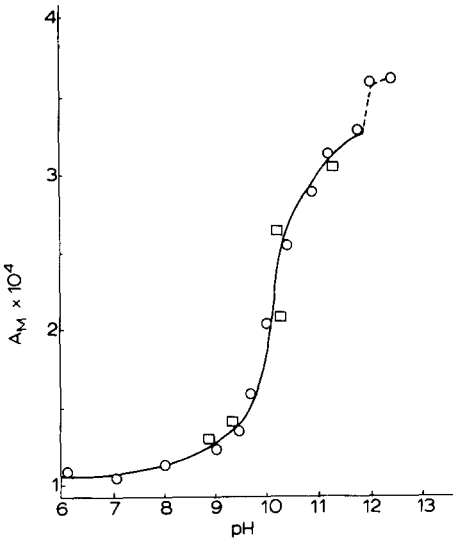


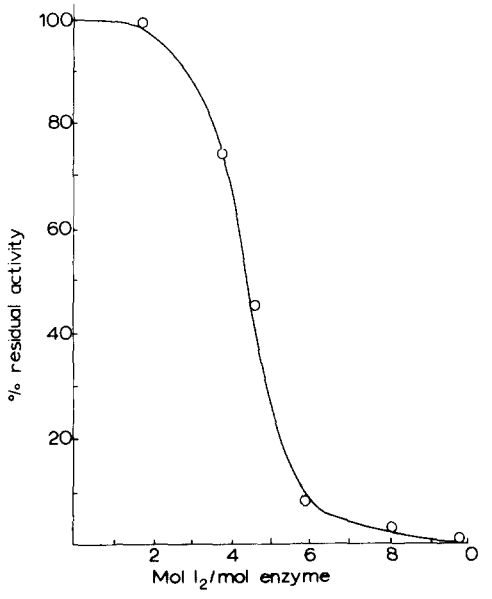
Fig. 1. The spectrophotometric titration of mitochondrial L-malate:NAD^+ oxidoreductase obtained by a plot of the molar extinction coefficient (A_M) as a function of pH. The absorbance was measured at 295 nm. The initial pH was 6.0 and the pH at the end of the reversible portion of the curve (solid line) was 12.0. The denatured portion of the curve is represented by the broken line. The forward reaction (○) was carried out by additions of 1.0 M NaOH while the reverse reaction (□) was produced by additions of 1 M HCl at various pH values. Concentrations of 1.0—2.0 mg/ml of 0.15 M KCl of the enzyme were used.

concentration 2.2 tyrosyl residues were nitrated. As the tetranitromethane concentration was further increased, the activity decreased, and the number of tyrosines nitrated were also slightly increased until a 1460 M excess produced zero activity and a total of 3.4 nitrated tyrosine residues. The error in evaluating the number of nitrated tyrosines was ±0.2. Nitration of the enzyme under the above conditions in the presence of NAD^+ and low concentrations of oxaloacetate (where this substrate would not act as an inhibitor) yielded a 98%

TABLE I

NITRATION IN THE PRESENCE AND ABSENCE OF NAD^+ AND OXALOACETATE

Molar excess of tetranitromethane (no NAD^+ and oxaloacetate)	% Residual activity after 2 h	Number of nitrated Tyrosine residues
17	100	0
29	100	0
100	100	1.5
146	78	2.2
291	8	3.1
1460	0	3.4
(NAD^+ and oxaloacetate)		
1460	98	0



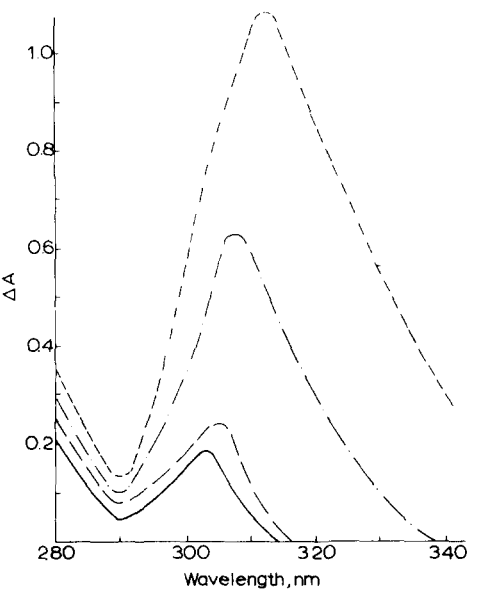


Fig. 2. Titration of enzyme with I_2 measured as a function of the percent residual activity of the enzyme. The reaction was carried out in 50 mM phosphate buffer (pH 7). Amounts of I_2 were added to the enzyme and the reaction was assumed to be completed when the color disappeared.

Fig. 3. Ultraviolet difference spectra of I_2E (———), I_6E (— — —), I_8E (— · — · —) and $I_{14}E$ (· · · · · ·). The reference sample was at pH 2.5, while the test sample was at pH 11.5.

active molecule, suggesting that the oxaloacetate and NAD^+ afforded protection to the site of nitration.

From 4,4-bis-dimethylaminomethyl carbinol titration, the number of sulfhydryl groups in the native and nitrated enzyme were observed to be 13.9 ± 0.3 and 14.1 ± 0.3 thiol groups, respectively. The variation was obtained by triplicate measurements. The number of titratable cysteinyl residues was found to be in agreement with the reported literature value of 14 [25] and it agreed with the supposition that none of the cysteine side chains were oxidized during the nitration of the tyrosines.

The results from the iodination of the tyrosyl residues as a function of enzyme activity, are presented in Fig. 2. No inhibition was observed when 2 mol I_2 were reacted per mol enzyme. As further additions of I_2 were made, the activity decreased to near zero when 6 mol I_2 per mol enzyme was added.

Ultraviolet difference spectra at pH 11.5 vs. pH 2.5 were run on four iodinated species (Fig. 3). The protein containing 2 mol I_2 (I_2E) per mol enzyme (solid line) shows a maximum at 303 nm which corresponds to the λ_{max} for monoiodinated tyrosine [26], while the curve for the 14 mol I_2 ($I_{14}E$) per mol enzyme (dotted line) has a maximum at 312 nm which corresponds closely with the λ_{max} of 311 nm for diiodinated tyrosine. Addition of I_2 in an amount greater than 12 mol per mol enzyme resulted in unreacted I_2, and the spectrophotometric curves were identical with the $I_{14}E$ specie.

The enzyme, either native, nitrated, or nitrated in the presence of NAD^+ and

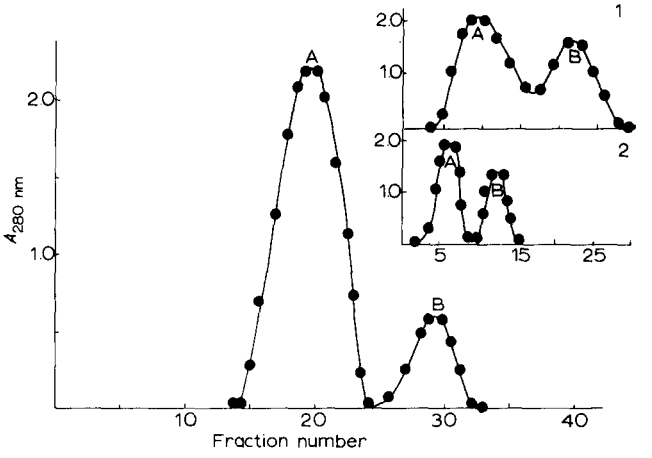


Fig. 4. Sephadex G-25 elution pattern of the cyanogen bromide hydrolysis of native enzyme. The CNBr hydrolyses of both nitrated species were the same as the native molecule. Inset 1 is the Sephadex G-50 elution pattern of peak A from G-25 for the native molecule, while inset 2 is the G-75 elution pattern of peak A from G-50. Similar Sephadex separations were run on the two nitrated species and the elution patterns were the same as those in insets 1 and 2.

oxaloacetate, which had been hydrolyzed with CNBr, was initially chromatographed on a Sephadex G-25 column previously equilibrated in 30% acetic acid. The elution pattern of any of the hydrolyzates (they were essentially the same) is shown in Fig. 4. The first peak contained four peptides as observed from

TABLE II

AMINO ACID ANALYSIS OF PEAK A FROM SEPHADEX G-75 OF THE NATIVE, NITRATED AND NAD^+-OXALOACETATE BOUND NITRATED ENZYME

All analyses were performed in duplicate and the variation between tests were within 10%.

Amino acid	Number of residues per mol peptide		
	Native	Nitrated	NAD^+-oxaloacetate nitrated
Ala	6.4	6.7	6.2
Arg	2.2	1.9	1.8
Asp	5.8	5.4	5.5
Glu	6.4	6.1	5.9
Gly	6.8	6.0	6.7
His	1.5	1.1	1.2
Ile	3.7	4.0	4.2
Leu	7.8	7.0	7.2
Lys	6.1	5.8	5.8
Phe	1.0 *	1.0	0.8
Pro	4.6	4.2	4.3
Ser	3.0	3.5	3.3
Thr	4.3	4.6	4.7
Tyr	1.6	0.1	1.5
Val	5.6	5.9	5.2
Total	66.8	63.6	64.3

* Standard.

polyacrylamide-gel electrophoresis. The second peak contained three peptides as evidenced by high-voltage electrophoresis. These latter peptides were obviously quite small due to the inability of the Coomassie Blue to stain them on the gels. Application of the proteins comprising the first peak (A) to a Sephadex G-50 column produced two peaks, as shown in inset (1) of Fig. 4. Polyacrylamide-gel electrophoresis of these peaks indicated two peptides in each. When the proteins from the first peak off of the G-50 column were chromatographed on a Sephadex G-75 the expected two peaks were observed, as shown in inset (2) of Fig. 4, and polyacrylamide-gel electrophoresis of each, indicated one peptide per peak. Similar results were obtained when the second peak from the G-50 column was chromatographed on the same G-75 column.

The A peptides obtained from the G-75 chromatography of the native enzyme, and the enzymes nitrated in the presence and absence of NAD^+ and oxaloacetate, were subjected to amino acid analysis, and the results are shown in Table II. Nitrotyrosine residues were not detected in any of the other peptides by spectroscopic absorption measurement at 381 nm.

Discussion

Reaction of the tyrosyl residues under three different sets of conditions (pH titration, iodination and nitration) produced results which suggests that the accessibility of amino acid side chains to the solvent depended greatly upon the reagent used in the studies. Potentiometric titration indicated that only one tyrosyl residue did not have access to the solvent, and was only titratable when the enzyme was irreversibly denatured. Since the other 8—9 phenolic hydrogens titrated at normal pK values one would assume that they were near the surface of the molecule. However, all the tyrosyls titrate normally in bovine serum albumin even though several are buried in the hydrophobic regions of the molecule. From the iodination data it appeared as if only seven of the tyrosyls could react with the iodine reagent. This was based on the observation that addition of 14 mol or more or iodine per mol of enzyme produced the same number of diiodotyrosine residues. Although the precision of these experiments were not as good as those of the pH titrations, there was a strong suggestion of differences in tyrosyl accessibility to the solvent. The nitration experiments showed that under the conditions in which tetranitromethane was added only four tyrosyls were accessible to the nitrating reagent, even under extremely excessive nitrating concentrations. These experiments suggested that the tyrosyl side chains are in various states of solvent exposure.

Data from the experiments resulting from the nitration or iodination of the enzyme supported previous evidence [10] which indicated that there may be one or two tyrosines which affect the biological activity. Interpretation of the iodination data indicated that one or two tyrosyl residues, which were extremely reactive, were probably not involved with catalysis, since there was no loss of activity. It had been shown [27] that the rate of monoiodination of tyrosyl residues was significantly faster than diiodination, and since the difference spectral data in Fig. 2 showed a peak at 303 nm for the addition of 2 mol I_2 per mol enzyme (which was identical to the λ_{max} of monoiodotyrosine) it is suggested that two rapidly reacting tyrosines have been monoiodinated. Further

iodination showed a progressive loss of activity suggesting that the newly iodinated phenolic sidechains have been implicated in biological activity.

Complimentary data supporting the iodination results have been shown by nitration with tetranitromethane. These experiments showed that significant activity was not lost until after 2 tyrosyl residues were nitrated. When 3—4 residues were nitrated, the enzyme was completed inactivated. Thus, 1—2 tyrosyl side chains have been implicated in the activity of the enzyme. The method of nitration was similar to that of Hum et al. [28], who indicated that no sulfhydryl residues were oxidized in their treatment of gonadotropin with tetranitromethane. It was also observed in the present studies that titration of the nitrated molecule with 4,4-bis-dimethylaminodiphenyl carbinol showed no oxidized cysteinyl residues. This evidence supported the original assumption that the loss of activity was due to the modification of the tyrosyl side chains.

A CNBr-mapping experiment was performed on the native, nitrated and substrate protected and nitrated enzymes in order to determine the aproximate location of the active tyrosyl residue. This protection of the enzyme was assumed to be taking place at the active site since there was no evidence to show that NAD^+ or oxaloacetate bind elsewhere. Resolution of the CNBr peptides was effected by the use of Sephadex-gel columns. After separation of the various peaks from each of the Sephadex chromatographies it was observed (by spectrophotometric measurement) that only the A peaks from G-25, G-50 and G-75 contained nitrotyrosine in the nitrated enzyme derivatives. The nonnitrated enzyme also yielded a peak at the same location as the nitrated derivatives.

Amino acid analysis of peak A from each of the Sephadex G-75 chromatographies showed that one or two tyrosyl residues in each of nitrated species had been altered. Unfortunately, the nitrotyrosines could not be located in the analysis, and either they were destroyed during the acid hydrolysis or they combined with one of the other amino acids. Since the nitro group lowers the pK of the phenolic hydrogen one would expect this ionization to be on the acid side of the tyrosine peak; nevertheless they were not observed.

These results suggested that the nitration of tyrosine inactivated the enzyme since no other amino acid appears to be affected by the tetranitromethane reagent. Very probably, both nitrated residues appear in a 60—70 amino acid sequence per subunit (Table II) assuming that there are two identical subunits per enzyme molecule [16,29]. Recently, a sequence was reported [30] (Val-Ser-Val-Pro-Ile-His-Gly-Gly-Val-Ala-Gly-Lys), which contained a reactive histidine, but not a tyrosyl residue; however, from the amino acid composition given in Table II it is possible that the sequence containing the reactive tyrosine was an extension of this chain.

From the iodination data, it has been suggested that only one of these tyrosines play a role in the activity of the enzyme. The actual function of the tyrosyl residue(s) cannot be elucidated from the present information. Since eight of the residues titrated normally, and the other one remained unionized until the molecule was denatured, it was unlikely that the residue was involved in a transitional change during activation as had been expressed in the case of elastase [31]. It was, therefore, likely that the nitrotyrosine (pK 6.9) and the iodotyrosine ($pK_{monoiodo}$ 8.0 and pK_{diiodo} 6.5) were causing a delocalizing of

charges either at or near the active site, either by enhancing the ionization of the phenolic proton or by increasing the charge density due to the high electronegativity of the attached group. If this was true then the phenolic proton could be involved in a critical hydrogen bond or could possibly act as a proton donor during catalysis.

Acknowledgement

We would like to thank Dr. J. Aull for the use of the spectrophotometers and Dr. B. Dunlap (University of S. Carolina) for use of the Beckman Model 119 C amino acid analyzer.

References

1 Attassi, M.Z. and Habeeb, F. (1969) Biochemistry 8, 1385—1393
2 Menendez, C.J. and Herskovits, T.T. (1969) Biochemistry 8, 5052—5059
3 Hjelmgren, T., Arvidsson, L. and Larsson-Raznikiewicz, M. (1976) Biochim. Biophys. Acta 445, 342—349
4 Cramer, J.L. and Neuberger, A. (1943) Biochem. J. 37, 302—310
5 Herskovits, T.T. and Laskowski, Jr., J. (1968) J. Biol. Chem. 243, 2123—2129
6 Sokolovski, M., Riordon, J.F. and Vallee, B.L. (1966) Biochemistry 5, 3582—3589
7 Cha, C.Y. and Scheraga, H.A. (1963) J. Biol. Chem. 258, 2958—2971
8 Gregory, E.M., Yost, F.J., Rohrbach, M.S. and Harrisson, J.H. (1971) J. Biol. Chem. 246, 5491—5497
9 Anderton, B.H. (1970) Eur. J. Biochem. 15, 562—568
10 Siegel, L. and Ellison, J.S. (1971) Biochemistry 10, 2856—2862
11 Wolfe, R.G. and Neilands, J.B. (1956) J. Biol. Chem. 221, 61—69
12 Kitto, G.B. and Kaplan, N.O. (1966) Biochemistry 5, 3966—3980
13 Gabriel, O. (1971) Methods Enzymol. 22, 565—578
14 Silverstein, E. and Sulebele, G. (1969) Biochemistry 8, 2543—2550
15 Goodwin, T. and Morton, F.A. (1946) Biochem. J. 40, 628—632
16 Noyes, B.E., Glatthaar, B.E., Garavelli, J.S. and Bradshaw, R.A. (1974) Proc. Natl. Acad. Sci. U.S. 71, 1334—1340
17 Siegel, L. and Englard, S. (1962) Biochim. Biophys. Acta 64, 101—110
18 Tanford, C. and Roberts, Jr., G.L. (1952) J. Am. Chem. Soc. 74, 2509—2515
19 Lowry, D.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
20 Friedman, M.E., Musgrove, B., Lee, K. and Teggins, J.E. (1971) Biochim. Biophys. Acta 250, 286—296
21 Humphries, B.A., Rohrbach, M.S. and Harrison, J.H. (1973) Biochem. Biophys. Res. Commun. 50, 493—495
22 Gross, E. and Witkop, B. (1961) J. Am. Chem. Soc. 83, 1510—1511
23 Hermans, J. (1962) Biochemistry 1, 193—196
24 Tachibani, A. and Murachi, T. (1966) Biochemistry 5, 2756—2762
25 Rohrbach, M.S., Humphries, B.A., Yost, Jr., F.J., Rhodes, W.G., Boatman, S., Hiskey, R.G. and Harrison, J.H. (1973) Anal. Biochem. 52, 127—142
26 Woody, R.W., Friedman, M.E. and Scheraga, H.A. (1966) Biochemistry 5, 2034—2042
27 Mayberry, W.E., Rall, J.E., Berman, M. and Bertole, D. (1965) Biochemistry 4, 1965—1972
28 Hum, V.G., Knipfel, J.E. and Mori, K.F. (1974) Biochemistry 13, 2359—2364
29 Gerdin, R.K. and Wolfe, R.G. (1969) J. Biol. Chem. 244, 1164—1168
30 Banasjak, L.J. and Bradshaw, R.A. (1975) in The Enzymes (Boyer, P., ed.), Vol. 9, p. 393.
31 Gorbunoff, M.J. and Timasheff, S.N. (1972) Arch. Biochem. Biophys. 152, 413—418