Vol. 81, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1978 Pages 28-34

Structural Changes in Metalloenzyme in the Course of Metal Substitution: Carboxypeptidase B

Nava Zisapel

Department of Biochemistry
The George S. Wise Center for Life Sciences
Tel-Aviv University, Tel-Aviv, Israel

Received December 8,1977

SUMMARY

Native and zinc reconstituted carboxypeptidase B were nitrated with tetranitromethane. The inactivation of the reconstituted enzyme was faster than that of the native enzyme and was accompanied by the formation of a considerable amount of enzyme dimers. The inactivation and dimerization reflected changes in the reactivity of active site tyrosine residue(s), thus indicating microenvironmental changes which occur during metal substitution. The change in tyrosine reactivity could be correlated with the residence of the enzyme in the metal-free state.

Substitution of metal ions at the active site of metalloenzymes is considered among the most mild and selective procedures employed in the studies of structure function relationship in these enzymes (1-8). The catalytic function is generally preserved in such substitutions, this being interpreted as indicating that the overall protein conformation is not measurably altered. To investigate the occurance of ultrastructural differences in the microenvironment of the metal at the active site of carboxypeptidase B (EC 3.4.2.2)* upon removal and restoration of a metal ion, nitration of an active site tyrosyl residue was performed in the native and reconstituted zinc enzymes. Native CPB is known to be inactivated by tetranitromethane as the result of the nitration of an active site tyrosyl residue (Tyr 248)(9). The selective nitration of Tyr 248 has been attributed to the lower pK of the phenolic hydroxyl of this residue arising most probably from the proximity of a positive charge - either the active site zinc ion or an arginine residue (10,11). TMN modification could thus serve as a tool for the detection of topographical or microenvironmental changes in the area of the active site, since nitration of Tyr 248 would be sensitive to its state or position

* Abbreviated as CPB

0006-291X/78/0811-0028$01,00/0

relative to other active site constituents. The results presented here indicate that although catalytic activity of the enzyme is retained upon removal and restoration of the metal, structural changes in the active site are detected.

MATERIALS AND METHODS

Porcine CPB was prepared according to the method of Folk et al (12) and purified on Sepharose ε-amino-caproyl-D-arginine column (13).

All buffers were extracted with dithizone in CCl_4 to remove metal contamination. Apo and reconstituted CPB were prepared as previously described (10).

Nitration of CPB was performed as described (9) with 8 fold molar excess of TNM in Tris buffer, pH 8.0 (0.1M NaCl, 0.05M Tris.HCl) 25°, 60 minutes. The nitrated enzyme samples were fractionated through Sephadex G-75 with 0.1M NH_4HCO_3 as the eluent. Enzyme activity was assayed with t-butyloxycarbonyl-L-alanyl-L-alanyl-L-phenylalanine (t-Boc-$(ala)_2$-phe(L_3)) as described (11)(14). Oxidation with performic acid was performed by suspending the lyophilized enzyme in 1 ml performic acid for 2 hours at room temperature. The acid was then evaporated to dryness over sodium hydroxide pellets.

Amino acid analysis was performed in a Beckman Unichrome analyser (15) after acid hydrolysis of the protein (6N HCl,110° 22 hours). Sodium dodecyl sulfate (SDS) 10-20% polyacrylamide gradient slab gel electrophoresis was performed according to Laemmli (16) with 0.1% SDS in the running buffer. Samples were dissolved in SDS polyacrylamide sample buffer containing 1% SDS and 0.1M mercaptoethanol and boiled at 100° for 5 minutes prior to electrophoresis. Metal content was determined by atomic absorption spectroscopy (Varian Techtron AA5) and was found to be 0.96-0.98 g atom metal per mole enzyme.

Protein concentration was determined either by spectrophotometric measurement at 278 nm or by the procedure of Lowry et al (17).

RESULTS AND DISCUSSION

Removal of zinc ion from the active site of CPB by treatment with 4 mM o-phenanthroline at pH 5.5 (10) results in total loss of catalytic activity. Reconstitution of the apoenzyme with Zn^{2+} yields a fully active enzyme. The specific activities of the reconstituted enzyme preparations towards t-Boc-$(ala)_2$-phe were 150-160 μmoles hydrolyzed substrate/min/mg protein (i.e. 95-100% active as compared to the native enzyme). Affinity chromatography experiments of the reconstituted enzyme on sepharose-ε-aminocaproyl-D-arginine showed that all the protein was retarded by the column and could be eluted with 2M NaCl, 0.05M Tris.HCl buffer, pH 7.5 as the native enzyme (13).

The rate of inactivation of reconstituted CPB by tetranitromethane was faster than that observed for the native enzyme (Fig. 1). The inactivation followed a first order kinetics in the native but not in the reconstituted enzyme. In addition, nitration of the reconstituted

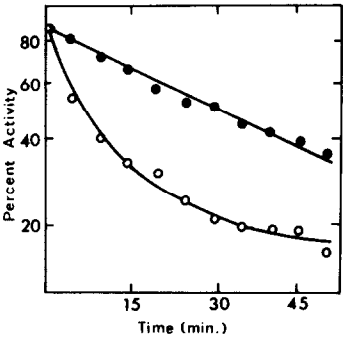

Fig. 1: Inactivation of native (•) and reconstituted (o) CPB by TNM. Activity was monitored as a function of time on 0.01M t-Boc-Ala-Ala-Phe (L_3) and expressed as percentage of the unmodified controls.

enzyme lead to the formation of a considerable amount of inactive high molecular weight material (Fig. 2)(Table 1). In the native enzyme and in native enzyme which has been dialysed against 0.1 mM Zn^{+2} in Tris-buffer pH 7.5, on the other hand, the TNM-induced inactivation was much slower and was not accompanied by extensive formation of the protein oligomers (10)(Table 1). The extensive formation of oligomers in the case of the reconstituted enzyme cannot be ascribed to the formation of disulfide bond(s) which results from oxidation of SH groups knows to be present in CPB (3) since oxidation of the nitrated enzyme oligomers with performic acid did not dissociate the protein aggregates. Moreover, the aggregates did not dissociate under the denaturing and reducing conditions of the SDS-polyacrylamide sample buffer. The combined data thus indicates that covalent bonds other than -S-S- are most probably involved in the cross linking process. The apparent molecular weights of the polymerized protein determined by SDS acrylamide gel electrophoresis were 35,000 for the monomer and 70,000 for the oligomer, hence suggesting dimerization. Both purified nitro-CPB preparations,monomeric and dimeric (Sephadex G-75, Fig. 2) were almost inactive towards the substrate used (Table 2); the faster inactivation of TNM treated reconstituted CPB could thus be related to more extensive formation of cross-linked inactive dimers (Table 1). Zinc and amino acid analyses of the monomeric and dimeric nitro-CPB were similar,differing only in nitrotyrosyl content: 1.1 nitrotyrosine in the dimer and 0.6 nitrotyrosine in the monomer. Change in tyrosine(s) reactivity is hence most probably involved in the extensive dimerization of the reconstituted enzyme. It should be noted in this

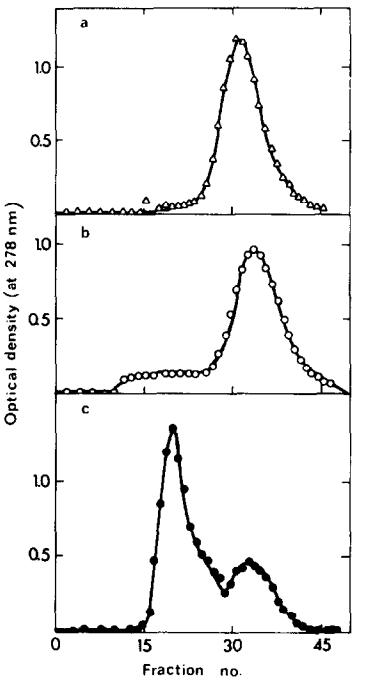

Fig. 2: Gel filtration of a) native CPB b) reconstituted CPB c) nitroreconstituted CPB (conditions of nitration: 2 mg/ml CPB, 8 fold molar excess of TNM, in 0.1M NaCl - 0.05M Tris-HCl, pH 8.0, 25°, 60 minutes. Activity of the modified enzyme was 25% of the unmodified control).

context that proteins have been shown to undergo cross-linking during nitration via oxidation of tyrosyl residues to form dityrosine (18)(19).

The presence of competitive inhibitors of CPB i.e. β-phenylpropionic acid or acetyl-D-arginine (10)(14) markedly reduced both inactivation and dimerization (Table 1). This implicates active site residue(s) in both processes. Intermolecular crosslinking through active site tyrosyl residues would be expected to take place if the latter are located on the surface of the protein molecule. The extent of dimer formation during nitration thus most probably reflects active site conformational changes related to the exposure of tyrosyl residue(s) during metal substitution.

Short treatment of CPB with o-phenanthroline at neutral pH did not alter the state of the tyrosyl residue as deduced from the rate of inactivation and the amount of dimers formed during nitration (Table 2). In addition, dialysis of the enzyme against high concentration of metal

Table 1: Nitration of Native and Reconstituted Zinc Carboxypeptidase B[a,b]

Enzyme	Inhibitor	After reaction	
		Activity of the unresolved enzyme, %[c]	Dimer formed, %[d]
Native CPB	-	46	15
Native CPB dialysed against 0.1 mM Zn^{+2}	-	42	16
Reconstituted Zn-CPB	-	15	64
Reconstituted Zn-CPB	Acetyl-D-Arginine (0.1M)	80	5
Reconstituted Zn-CPB	β-phenyl propionic acid (0.1M)	90	5

[a] The apoenzyme was prepared by treatment with 4 mM o-phenanthroline at pH 5.5 as described (10).

[b] Conditions for nitration: 2 mg/ml CPB, 8 fold molar excess TNM, in 0.1M NaCl - 0.05M Tris.HCl, pH 8.0, 25°, 60 minutes.

[c] Expressed as percentage of the unmodified controls.

[d] After reaction the dimeric and monomeric products were separated on Sephadex G-75 column. Dimer expressed as percentage of total protein eluted from the column.

ion did not cause a measurable degree of ultrastructural perturbation (Table 1). It should be stressed that the conditions employed for the preparation of the apoenzyme used for the experiments of Table 2 were much milder than those described in Table 1 which are frequently in use (i.e. pH 7.5, 30 min in Table 2, as compared to pH 5.5,75 hours in Table 1). The relation between the period that the enzyme spent in the apoenzyme state and the change in the reactivity of the active site tyrosyl residue(s) was examined as follows. The apoenzyme was prepared by incubation of the native enzyme with 1 mM of o-phenanthroline solution in Tris buffer, pH 7.5, at 0° for 30 min, followed by desalting on a

Table 2: Effects of TNM on Reconstituted CPB as Function of the Residence of the Enzyme in the Apo State[a,b,c]

Enzyme	Nitrotyrosine per mole enzyme	% dimer formed	% activity[d]		
			total	dimer	monomer
1. Native CPB	0.84	15	41	9.1	47
2. Reconstituted CPB[a]					
t = 0 minutes	0.99	18	49	10.0	57
t = 20 minutes	1.03	18	32	8.7	37
t = 60 minutes	1.1	29	20	9.0	25
t = 120 minutes	1.3	35	18	8.5	23
t = 1200 minutes	1.4	46	16	8.0	23

[a] Apoenzyme was prepared by treatment with o-phenanthroline for 30 min, at pH 7.5 as described in Results and Discussion.

[b] The enzyme was kept for different periods of time (t) as apoenzyme prior to its reconstitution with Zn^{+2}.

[c] Conditions for nitration: 2 mg/ml CPB, 8 fold molar excess of TNM, in 0.1M NaCl, 0.05M Tris.HCl, pH 8.0, 25°, 60 minutes.

[d] Expressed as percentage of the unmodified enzyme.

Sephadex G-25 column. The apoenzyme, eluted in metal-free Tris buffer, pH 7.5, was kept for different periods at 0° prior to its reconstitution. Equimolar concentrations of Zn^{+2} ions were then added, and the enzyme was kept for 2 hours at 4° and then reacted with TNM. The results shown in Table 2 indicate that increase of residence of CPB in the apoenzyme state was accompanied by an increase in the rate of inactivation of the reconstituted enzyme by TNM, the number of nitrotyrosine residues formed (determined by amino acid analysis) and the formation of dimers. These findings strongly suggest that the native conformation of CPB in the

apoenzyme form is preserved only for a finite period of time.

The procedure used for the preparaton of apoenzymes should hence be modified so as to conserve the active site in its native state. Furthermore, the use of several criteria, including chemical modifications of microenvironmentally-sensitive active site residues should be important for a more unequivocal interpretation of metal substitution experiments aimed at the ellucidation of the mechanism of action of metalloenzymes.

ACKNOWLEDGEMENT

This research was supported in part by the Israel Commission for Basic Research. I wish to thank Prof. Mordechai Sokolovsky for valuable discussions.

REFERENCES

1) Rickli, E.E., and Edsall, J.T. (1962) J. Biol. Chem. 237, PC 258-260.
2) Coombs, T.L., Omote, T., and Vallee, B.L. (1964) Biochemistry 3, 653-657.
3) Wintersberger, E., Neurath, H., Coombs, T.L., and Vallee, B.L. (1965) Biochemistry 4, 1526-1532.
4) Simpson, R.T., and Vallee, B.L. (1968) Biochemistry 7, 4343-4347.
5) Vallee, B.L., and Williams, R.J.P. (1968) Proc. Nat. Acad. Sci. USA. 59, 498-505.
6) Auld, D.S., and Vallee, B.L. (1970) Biochemistry 9, 4352-4356.
7) Zisapel, N., and Sokolovsky, M. (1973) Biochem. Biophys. Res. Commun. 53, 722-729.
8) Zisapel, N., Navon, G., and Sokolovsky, M. (1975) Eur. J. Biochem. 52, 487-492.
9) Sokolovsky, M. (1972) Eur. J. Biochem. 25, 267-273.
10) Shaklai, N., Zisapel, N., and Sokolovsky, M. (1973) Proc. Nat. Acad. Sci. USA. 70, 2025-2028.
11) Zisapel, N., Shaklai, N., and Sokolovsky, M. (1975) FEBS Letters 51, 262-265.
12) Folk, J.E., Piez, K.A., Carroll, W.R., and Gladner, J.A. (1960) J. Biol. Chem. 235, 2272-2277.
13) Sokolovsky, M. (1974) In: Methods in Enzymol. (Jacoby, W.B. and Wilchek, M. eds) pp.412 Academic Press, New York.
14) Zisapel, N., and Sokolovsky, M. (1972) Biochem. Biophys. Res. Commun. 46, 357-363.
15) Spackman, D.H., Stein, W.H., and Moore, S. (1958) Anal. Chem. 30, 1190-1196.
16) Laemmli, U.K. (1970) Nature 227, 680-685.
17) Lowry, O.H., Rosebrough, M.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
18) Aeschbach, R., Amado, R., and Neukom, H. (1976) Biochim. Biophys. Acta. 439, 292-301.
19) Williams, J. and Lowe, J.M. (1971) Biochem. J. 121, 203-209.