SYNCATALYTIC MODIFICATION OF CYTOPLASMIC ASPARTATE AMINOTRANSFERASE: IDENTIFICATION OF A PEPTIDE CONTAINING THE MODIFIED CYSTEINYL RESIDUE

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1. Introduction

Syncatalytic inactivation of cytoplasmic aspartate aminotransferase has first been found using tetranitromethane (TNM) as modifying reagent [1]. With TNM one sulfhydryl group per enzyme monomer is oxidised and concomitantly tyrosyl residues are nitrated [1–3] *. Recently, we have also demonstrated syncatalytic modification of one sulfhydryl group per monomer with selective thio reagents [5, 6]. In the present communication we report the syncatalytic modification of aspartate aminotransferase with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). Using this reagent it was possible to radioactively label one cysteinyl residue per monomer by displacement of the thionitrobenzoate (TNB) moiety by [^{14}C]cyanide. Combined CNBr and tryptic cleavages of the enzyme derivative labelled by this procedure and analyses of the resulting peptides demonstrated that the syncatalytic modification involves one specific sulfhydryl group. Amino acid composition and C-terminal analysis of the isolated radioactive peptide allowed its localisation in tryptic peptide 22 reported by Ovchinnikov et al. [7] as part of their amino acid sequence studies on cytoplasmic aspartate aminotransferase.

* Reexamination of the TNM reaction has indicated that thiol oxidation rather than tyrosyl nitration represents the actual syncatalytic modification, i.e. tyrosyl nitration might be a secondary reaction which depends on preceding sulfhydryl modification [4].

2. Experimental

The α-subform of cytoplasmic aspartate aminotransferase was isolated from pig heart according to the procedure of Banks et al. [8], as modified in our laboratory. Protein (monomer) concentration was determined spectrophotometrically using an absorptivity $\epsilon_{280} = 7.0 \times 10^4\ M^{-1}\ cm^{-1}$. This value is based on amino acid analyses, on coenzyme determinations, and on an assumed molecular weight of 46 500 per monomer [9]. The enzyme assayed according to the procedure of Karmen [10] had a specific activity of 310–350 units/mg.

The total thiol group content of aspartate aminotransferase was determined with 0.4 mM DTNB [11] in 6 M guanidine-HCl [5] or in 0.4% SDS–0.1 M sodium phosphate, pH 7.5. The rapidly reacting thiol groups of the native enzyme were titrated either with DTNB (0.4 mM) or with [^{14}C]*N*-ethylmaleimide (NEM) (2 mM) in 0.1 M sodium phosphate, pH 7.5. Syncatalytic modification of the enzyme was carried out in 0.5 M sodium phosphate, pH 7.5, with 12 mM DTNB in the presence of 70 mM glutamate, 2 mM α-ketoglutarate, 1 mM PLP, and 1 mM PMP, for 20 hr at room temperature. In these experiments the rapidly reacting thiol groups had been blocked beforehand by NEM. The excess of reagents and substrates was removed by gel filtration on a Sephadex G-25 column, equilibrated with 0.1 M sodium phosphate, pH 7.5. The displacement of the TNB-group with [^{14}C]cyanide (2 mM) [12] was carried out in 0.1 M sodium phosphate, pH 7.5, using the enzyme in the pyridoxamine form. The sulfhydryl groups unreactive under the conditions employed for syncatalytic modi-

fication were blocked with 2 mM NEM in 6 M guanidine-HCl–0.1 M sodium phosphate, pH 7.5. Excess reagent and denaturant were removed on a Sephadex G-25 column, equilibrated in 70% formic acid.

CNBr cleavage was carried out according to Gross and Witkop [13]. The resulting peptides were chromatographed on a 1.5 × 150 cm column of Sephadex G-75, equilibrated with 10% formic acid [14]. Peptide fractions were rechromatographed on a 0.9 × 150 cm column of Sephadex G-50 (superfine). After lyophilisation the radioactively labelled fractions were hydrolysed by TPCK-trypsin (2%, mole/mole) at 37° for 14 hr in 0.1 M NH_4HCO_3, pH 8.8. The tryptic peptides were fractionated on a 0.6 × 16 cm column of Dowex 50-X8 (AA-15 resin, Spinco) at 55° using a double linear pyridine–acetate gradient [15]. Subfractionation was carried out on a 0.9 × 140 cm column of Dowex 1-X2 at 35° with a continuous pyridine–acetate gradient [15]. Amino acid analyses were performed on a Beckman 120 B analyzer after hydrolysis with 6 N HCl for 22 hr at 110° in evacuated tubes. Digestions with carboxypeptidases A and B, 4% and 3% (mole/mole), respectively, were carried out at 37° in 0.1 M Tris-Cl, pH 8.5.

3. Results

In denatured cytoplasmic aspartate aminotransferase 5 moles of thiol groups per monomer are titrated with DTNB (table 1). In the native enzyme only 2

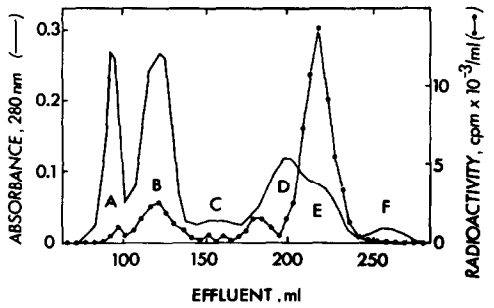

Fig. 1. Elution profile of the CNBr peptides of syncatalytically modified enzyme from chromatography on Sephadex G-75. Sulfhydryl groups I, II, IV, and V were blocked with NEM, thiol group III was labelled with [^{14}C]cyanide. Detailed modification conditions are given under Methods.

Table 1
Classes of thiol groups in cytoplasmic aspartate aminotransferase.

Enzyme	Reagent and conditions	Thiol groups modified (mole/mole)
Native	DTNB in guanidine-HCl	5.0[a]
	DTNB in SDS	4.9[a]
Native	DTNB in buffer	2.0[b]
	[^{14}C]NEM in buffer	1.8[c]
Modified with NEM in buffer	DTNB in glutamate plus α-ketoglutarate	1.0[a]
	[^{14}C]cyanide[d]	1.1[b]

The detailed reaction conditions and the methods of the quantitation of the modified thiol groups are given under Methods.

[a] Mean of 6 experiments.
[b] Mean of 4 experiments.
[c] Mean of 2 experiments.
[d] [^{14}C]cyanide incorporated after displacement of TNB by cyanide.

thiol groups (hereafter denoted I and II) react rapidly with DTNB and NEM. In the presence of glutamate and α-ketoglutarate an additional thiol group (III) is syncatalytically modified using high concentrations of the reagent. Thiol groups IV and V remain unreactive under the conditions used for syncatalytic modification. With thiol groups I and II modified the enzyme is fully active, whereas modification of thiol group III with DTNB decreases transaminase activity to about 5% of the initial value. Exposure of this derivative to either 140 mM 2-mercaptoethanol or

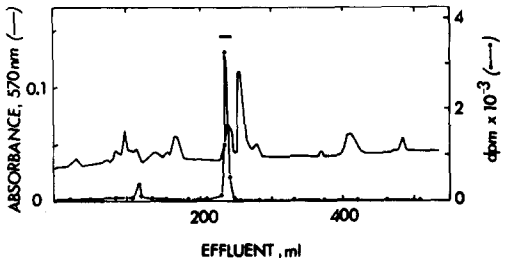

Fig. 2. Elution profile of the tryptic peptides of CNBr fragment E from chromatography on Dowex 50-X8. Thiol group III was labelled with [^{14}C]cyanide.

2 mM cyanide results in restoration of catalytic activity.

The specificity of the syncatalytic modification of sulfhydryl group III was examined by peptide analysis. The enzyme derivative employed for these experiments was blocked with NEM at thiol groups I and II, syncatalytically modified at thiol group III with DTNB, labelled with [^{14}C]cyanide by displacement of the incorporated TNB-moiety, and blocked by NEM at thiol groups IV and V after denaturation. Following CNBr cleavage and peptide separation the [^{14}C]label was found predominantly in one fraction (fraction E, fig. 1). Fraction E was rechromatographed and hydrolysed with trypsin; the peptides were fractionated on Dowex 50-X8 (fig. 2). Again, the radioactivity was located in one fraction which was further purified by chromatography on Dowex 1-X2 to yield an electrophoretically homogeneous peptide (m=0.38, pH 6.5 [16]) with the composition of 1.0 Lys, 1.6 Thr, 1.0 Leu, 1.1 Gly, 1.0 CN-Cys. The cyclised ninhydrin-negative cysteine derivative (2-iminothiazolidine-4-carboxylic acid) was identified and quantitated by scanning the effluent of the amino acid analyzer for radioactivity. Digestions of the peptide with carboxypeptidase B and A released 1.0 equivalent of Lys and 0.7 equivalent of Thr, respectively.

4. Discussion

The determination of a total of 5 sulfhydryl groups per monomer (table 1) is in agreement with recent investigations from other laboratories [8, 17–20]. However, in contrast to the findings of Braunstein [17] and Cournil and Arrio-Dupont [20] that 3 thiol groups may be modified prior to affecting transaminase activity the present investigation indicates that only 2 groups (I and II) can be blocked by either DTNB or NEM without loss of activity. Recently, the same observation was reported by Torchinsky and Sinitsyna [21] and Stankewicz et al. [18].

In the presence of glutamate and α-ketoglutarate one additional cysteinyl residue reacts with either DTNB or NEM [5]. This syncatalytic modification decreases the activity to approx. 5% of the initial value. The reactivation of the DTNB-modified enzyme by 2-mercaptoethanol further supports the conclusion that syncatalytic inactivation is due to modification of a thiol group.

Several investigators have postulated that an essential thiol group participates in catalysis and/or maintenance of the active conformation of aspartate aminotransferase [17, 18, 21–23]. However, the present finding that the enzyme derivative blocked with cyanide at thiol group II is enzymatically active strongly implies that this sulfhydryl group is not directly involved in these processes, though substituents bulkier than cyanide may impair the transaminase activity. On the other hand, the syncatalytic changes of this critically located group render it a probe for conformational changes of the enzyme in the covalent phase of catalysis.

The specificity of the syncatalytic modification is documented by combined CNBr and tryptic cleavages of an enzyme derivative labelled with [^{14}C]cyanide (figs. 1, 2)**. One peptide containing the radioactive label was isolated. Amino-acid analysis and carboxypeptidase digestions of this peptide indicate the sequence (Cys, Thr, Leu, Gly)–Thr–Lys which coincides with that of the C-terminal part of tryptic peptide 22 reported by Ovchinnikov et al. [7]:

CNBr cleavage
↓
peptide 22: Ile–Asn–Met–Cys–Gly–Leu–Thr–Thr–Lys
[^{14}C]peptide: (CN-Cys,Gly,Leu,Thr)–Thr–Lys

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References

[1] P. Christen and J.F. Riordan, Biochemistry 9 (1970) 3025.
[2] W. Birchmeier and P. Christen, Experientia 27 (1971) 727 (Abstract).

** Double modification experiments combined with analysis of the CNBr peptides indicates that DTNB, NEM and TNM all syncatalytically modify the same sulfhydryl group viz. thiol group III in CNBr peptide E. Of the remaining 4 thiol groups of aspartate aminotransferase one of the unreactive groups (IV or V) is found in CNBr peptide A and the other 3 are located in peptide B [4].

[3] O.L. Polyanovsky, T.V. Demidkina and C.A. Egorov, FEBS Letters 23 (1972) 362.
[4] W. Birchmeier, K.J. Wilson and P. Christen, in preparation.
[5] W. Birchmeier and P. Christen, FEBS Letters 18 (1971) 209.
[6] W. Birchmeier, K.J. Wilson and P. Christen, 8th FEBS-Meeting (1972) Amsterdam (Abstract).
[7] Yu.A. Ovchinnikov, A.A. Kiryushkhin, Ts.A. Egorov, N.G. Abdulaev, A.P. Kiselev, N.N. Modyanov, E.V. Grishin, A.P. Sukhikh, E.I. Vinogradova, M.Yu. Feigina, N.A. Aldanova, V.M. Lipkin, A.E. Braunstein, O.L. Polyanovsky and V.V. Nosikov, FEBS Letters 17 (1971) 133.
[8] B.E.C. Banks, S. Doonan, A.J. Lawrence and C.A. Vernon, European J. Biochem. 5 (1968) 528.
[9] N. Feliss and M. Martinez-Carrion, Biochem. Biophys. Res. Commun. 40 (1970) 932.
[10] A. Karmen, J. Clin. Invest. 34 (1955) 131.
[11] G.L. Ellman, Arch. Biochem. Biophys. 82 (1959) 70.
[12] T.C. Vanaman and G.R. Stark, J. Biol. Chem. 245 (1970) 3565.
[13] E. Gross and B. Witkop, J. Am. Chem. Soc. 83 (1961) 1510.
[14] T. Watanabe and H. Wada, Biochem. Biophys. Res. Commun. 43 (1971) 1310.
[15] R.A. Bradshaw, W.H. Garner and F.R.N. Gurd, J. Biol. 244 (1969) 2149.
[16] R.E. Offord, Nature 211 (1966) 591.
[17] A.E. Braunstein, FEBS Symp. 18 (1970) 101.
[18] M.J. Stankewicz, S. Cheng and M. Martinez-Carrion, Biochemistry 10 (1971) 2877.
[19] Yu.M. Torchinsky, personal communication.
[20] J. Cournil and M. Arrio-Dupont, Biochem. Biophys. Res. Commun. 43 (1971) 40.
[21] Yu.M. Torchinsky and N.I. Sinitsyna, Molekulyarnaya 4 (1970) 256.
[22] O.L. Polyanovsky and Yu.M. Torchinsky, in: Chemical and Biological Aspects of Pyridoxal Catalysis, eds. E.E. Snell et al. (Pergamon Press, 1963) p. 157.
[23] N.E. Vorotniskaya, G.F. Lutovinova and O.L. Polyanovsky, in: Pyridoxal Catalysis, Enzymes and Model Systems, Vol. 35, eds. E.E. Snell et al. (Interscience, New York, 1968) 131.