THE POSITION OF AN ESSENTIAL TYROSINE RESIDUE IN THE POLYPEPTIDE CHAIN OF ASPARTATE TRANSAMINASE

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1. Introduction

Cytoplasmic aspartate transaminase (EC 1.6.1.1) treated with tetranitromethane is known to suffer partial loss of activity [1]; almost complete inactivation under special conditions is accompanied by selective modification of tyrosine residues in the enzyme [2,3]. Here we present data showing that only one tyrosine residue is readily susceptible to nitration and its position in the polypeptide chain is determined by comparing the composition of the nitrotyrosyl peptides and the known sequences of the tryptic peptides from aspartate transaminase.

2. Experimental

The experiments were performed on cytoplasmic aspartate transaminase from pig heart muscle. The enzyme was purified as previously described [4] with minor modifications. The molecular weight of one polypeptide chain of this transaminase is about 47,000 (that of the dimeric structure is 94,000). One chain contains 13 tyrosine and 5 cysteine residues. The pure transaminase had the following optical characteristics: $E^{1mg}_{430} = 0.135$ at pH 5.2; $E^{1mg}_{360} = 0.165$ at pH 8.3. The concentration of protein was established spectrophotometrically ($E^{1mg}_{280} = 1.40$); its enzymic activity was measured by direct spectrophotometry according to Jenkins [5], and the thiol groups were determined by Boyer titration [6] in 1% sodium dodecylsulphate. The content of nitrotyrosine in the protein was calculated from spectral data (the molar extinction of nitrotyrosine at 428 nm being 4100) [7].

Thiol groups of transaminase were shown to undergo partial oxidation on exposure to tetranitromethane. It is also known that alkylation of non-essential thiol groups of transaminase does not alter the catalytic activity and physico-chemical properties of the enzyme. To minimize thiol group oxidation we treated the original aspartate transaminase with iodoacetate. All experiments were performed with catalytically active carboxymethylated holoenzyme containing only two titratable thiol groups.

Aspartate transaminase was nitrated with a 15-fold excess of tetranitromethane with continuous gentle stirring in the presence of a substrate pair (1.75 mM oxoglutarate + 70 mM L-glutamate, pH 7.5, 2 hr, 20°), i.e. under conditions resembling those of "syncatalytic" modification of the enzyme as developed by Christen [2]. Residual activity and the amounts of nitrotyrosine formed were determined in aliquots of the reaction mixture in the course of incubation; activity decrease was found to correlate with the formation of nitrotyrosine. When activity dropped to 2.3%, nitration was stopped by gel filtration of the sample through Sephadex (G-50, fine). The nitrotyrosine content of

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modified transaminase amounted to 0.81 residues per subunit. Nitrated transaminase had two absorption maxima, one at 428 nm (nitrotyrosine absorption), and the other at 330 nm which could be assigned to protein-bound pyridoxamine-5′-phosphate (PMP) [2]. Unlike native transaminase, the nitrated enzyme was not reconverted into the pyridoxal-5′-phosphate (PLP) form upon addition of oxoglutarate. This result was in agreement with the data of Christen and Riordan [2] and showed the enzyme to have lost the capacity to effect an essential step of the catalytic cycle.

After nitration, one of the two thiol groups had disappeared. Recently Christen reported the syncatalytic alkylation by *N*-ethylmaleimide of thiol groups in aspartate transaminase [8]. In a special series of experiments we demonstrated that the same cysteine residue is modified in both syncatalytic procedures. These data will be discussed elsewhere [9].

The nitrated enzyme was carefully dialyzed against distilled water, the solution was adjusted to pH 3.5 and denaturated on a water bath (100°, 3 min). After repeated dialysis the enzyme protein was digested with trypsin pretreated with chymotrypsin inhibitor (L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone).

An aliquot of the tryptic digest was subjected to peptide mapping. On treatment with gaseous ammonia two bright and two faint yellow spots developed (fig. 1). Approximately 45 peptides were developed on the same map with ninhydrin.

It should be emphasized that when transaminase was nitrated under the same conditions in the absence of substrates, only negligible amounts of nitrotyrosine were found (about 0.1 eq. per subunit). In this case inactivation was less than 10%, and no nitrotyrosine-containing peptides could be detected on the peptide maps.

To isolate the nitrotyrosyl peptides we subjected the tryptic digest to electrophoresis for 2.5 hr (acetic acid–pyridine, pH 3.4; 30 $V \cdot cm^{-1}$), developed the electrophoregram with ammonia and then paper-chromatographed the isolated nitrotyrosyl peptides

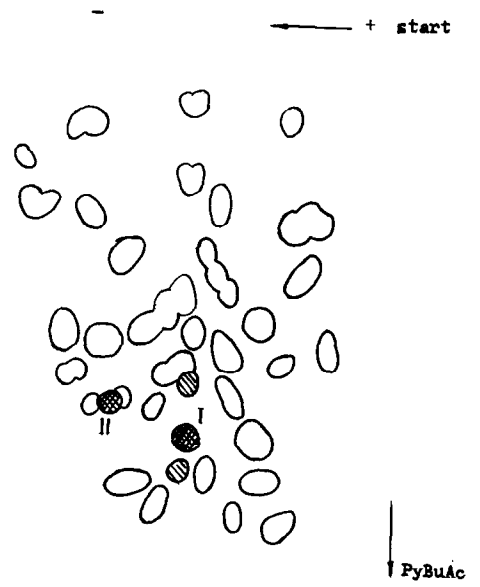

Fig. 1. Peptide map of tryptic digest of syncatalytically nitrated aspartate transaminase.

in the system: pyridine–butanol–acetic acid–water. Final purification was achieved with high-voltage electrophoresis (acetic acid–formic acid; pH 1.9; 80 $V \cdot cm^{-1}$; 30 min). The electrophoretic and chromatographic behaviour of peptides 1 and 2 was similar to that of substances I and II on the fingerprint (fig. 1).

After electrophoresis the peptides were eluted with 0.1% ammonia and lyophilized. Part of the material in each case was hydrolyzed under standard conditions (5.7 N HCl; 105°; 24 hr). The amino acid content was determined on the Bio-Cal (Munich) Model BC 201 analyzer.

3. Results and discussion

The amino acid composition of peptide II was as follows (μmoles): Lys 0.045 (1), Arg 0.045 (1), Asp 0.052 (1), Gly 0.099 (2), Ala 0.052 (1), Val 0.085 (2),

TA-26-I	Arg↓–Lys↓–Val–Asn–Leu–Gly–Val–Gly–Ala–Tyr–Arg–
Peptide II	Lys–Val–Asn–Leu–Gly–Val–Gly–Ala–Tyr(NO_2)–Arg–
Peptide I	Val–Asn–Leu–Gly–Val–Gly–Ala–Tyr(NO_2)–Arg–

Scheme 1.

Leu 0.050 (1), 3-NO_2-tyrosine (1). (Traces of Ser, Thr, Glu, Phe, His in quantities less than 0.005 μmoles each, i.e. less than 0.1 residue were detected). The composition of peptide I is the same as that of peptide II except for one lysine residue. It should be noted that both peptides contained small amounts of tyrosine.

Comparison of our data with the partial structure of aspartate transaminase reported previously [10] allows us to conclude that peptide II is identical to peptide TA-26-1, and peptide I is a part of peptide TA-26-1 (see scheme 1 on previous page).

Thus two nitrotyrosine peptides apparently originated from the cleavage of the peptide chain with trypsin at the arginine residue and at the neighbouring lysine residue (as marked with arrows).

The amino acid composition of all other tyrosyl peptides obtained from aspartate transaminase differs from the amino acid composition of the nitrotyrosine peptides isolated by us. Hence it follows that only one residue of tyrosine is modified on syncatalytic nitration of the transaminase polypeptide chain.

Finally we nitrated apotransaminase with a 15-fold excess of tetranitromethane (see [3]). The apoenzyme nitrated in this manner contained 1.1 eq. of nitrotyrosine (measured spectrophotometrically). Only 2.5% of the original activity was recovered in the nitrated apoenzyme upon addition of PLP. The nitrated enzyme was digested with trypsin. Examination of peptide maps in this case provided evidence that the same tyrosine residue was nitrated in apoenzyme and in holoenzyme.

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