IDENTIFICATION OF THE FUNCTIONALLY IMPORTANT CYSTEINYL RESIDUE IN PIG HEART ASPARTATE AMINOTRANSFERASE

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1. Introduction

L-Aspartate:2-oxoglutarate aminotransferase (EC 2.6.1.1) from pig heart cytosol consists of two identical subunits of molecular weight about 46,500 [1]. Each subunit has 5 thiol groups [2–5]: two exposed SH groups readily modified by iodoacetic acid with no decrease of activity, two fully buried SH groups inaccessible to thiol reagents in the native enzyme, and one relatively non-reactive, functionally important SH group, which can be selectively blocked by *p*-chloro-mercuribenzoate (*p*CMB), with 95% inactivation of the enzyme, after previous alkylation of the two exposed SH groups. It has been shown that selective mercaptidation of this SH group leads to considerable impairment of the enzyme's affinity for substrate analogues [5]. From the recent findings of Birchmeier and Christen [6] it appears that aminotransferase is similarly 95% inactivated when the functionally important SH group is subjected to "syncatalytic" alkylation with *N*-ethylmaleimide in the presence of a substrate pair. Modification of the functional SH group may be of critical significance for activity as a consequence of its proximity to the active site. We have achieved selective labeling of this group by syncatalytic alkylation with *N*-ethyl[^{14}C]-maleimide in the presence of glutamate and α-ketoglutarate, We have also carried out alkylation of partly and fully buried SH groups of the enzyme with iodo[^{14}C]acetic acid in 8 M urea and isolated radioactive tryptic peptides; one of these proved identical (in amino acid composition and nature of the N-terminal residue) with the labeled peptide isolated after syncatalytic alkylation of the enzyme with *N*-ethyl[^{14}C]maleimide. All experiments were performed on aminotransferase with the two exposed SH groups preliminarily blocked by "cold" iodoacetic acid.

2. Materials and methods

Aspartate aminotransferase from pig heart cytosol was purified as previously described [5, 7]. Protein was estimated spectrophotometrically, taking $E^{1\%}_{1\,cm}$ as 14.0 at 280 nm. SH groups were determined with *p*CMB according to Boyer [8].

Carboxymethylation of the exposed two SH groups in native enzyme was carried out by incubation with 50 mM "cold" iodoacetic acid in 0.1 M Tris-HCl buffer (pH 8.0, 24 hr, 4°); excess of iodoacetic acid was removed by dialysis. Such treatment does not alter the catalytic or physical properties of the enzyme.

N-Ethyl[1-^{14}C]maleimide (specific radioactivity 10.2 mCi/mmole) was purchased from Schwarz/Mann Co., USA, and diluted before use with carrier *N*-ethylmaleimide to a specific radioactivity 0.5 mCi/mmole. Iodo[2-^{14}C]acetic acid (from the Radiochemical Centre, Amersham, England; specific radioactivity 34 mCi/mmole) was diluted with carrier iodoacetic acid to a specific radioactivity of 4.1 mCi/mmole. Bovine trypsin, pretreated with diphenylcarbamyl chloride, was a product of PL-Biochemicals, Inc.

Digestion with trypsin was carried out in 0.5% NH_4HCO_3, pH 8.0, at 37°. Radioactive tryptic peptides were detected on paper by radioautography (using RT-2 X-ray films, exposure 24–72 hr); they were separated and purified by means of high-voltage

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electrophoresis (70–80 V per cm) and chromatography on Whatman No. 3 paper. Descending chromatography was done in the solvent system, pyridine–butan-1-ol–acetic acid–water (10:15:3:12). Peptides were eluted from the paper with 50 mM NH_4OH, freeze-dried and hydrolyzed (5.7 N HCl; 22 hr; 105°) in sealed evacuated tubes. Amino acid content was determined on the Bio-Cal Model BC 201 analyzer. N-Terminal residues of the peptides were determined by the dansyl procedure [9]; dansyl-amino acids were identified chromatographically on polyamide thin-layer sheets (Cheng-Chin Trading Co., Taiwan).

3. Results

3.1. *Reaction with N-ethyl[1-^{14}C]maleimide and isolation of labeled peptide*

"Syncatalytic" alkylation with labeled *N*-ethylmaleimide was carried out at room temp. in 50 mM Tris-HCl buffer, pH 7.5, under conditions resembling those described by Birchmeier and Christen [6]. The reaction mixture contained the enzyme (9 mg/ml), 140 mM sodium L-glutamate, 4 mM sodium α-ketoglutarate and 16 mM *N*-ethyl[^{14}C]maleimide. Incubation of the mixture for 18 hr lowered enzymic activity to 4% of the initial value and resulted in disappearance of one SH group as determined by *p*CMB titration. The modified enzyme was dialysed against several changes of 50 mM Tris-HCl buffer, pH 8.0, for 48 hr at 4°. After dialysis the enzyme was denatured in 8 M urea (3 hr at 25° and 5 min at 90°); urea was removed by dialysis against 0.001 N HCl at 4°.

The enzyme protein was digested with trypsin (1:50, w/w) for 24 hr; the clear solution was then adjusted with acetic acid to pH 2.8; a negligible precipitate formed upon acidification was separated by centrifugation, and the supernatant fraction, containing all the ^{14}C introduced into the enzyme, was freeze-dried. The digest was dissolved in 50% pyridine, streaked on paper and subjected to electrophoresis in pyridine–acetic acid buffer at pH 6.5. Radioautography revealed one single radioactive band corresponding in electrophoretic mobility to neutral amino acids. This band was cut out, sewn on to a fresh sheet of paper and submitted to chromatography*. Final purification of the radioactive peptide ($R_f \sim 0.5$) was achieved by electrophoresis at pH 3.5 and repetition of the chromatographic and electrophoretic runs. N-terminal group analysis revealed only isoleucine as the N-terminal residue. The amino acid composition of the peptide is:

Ile(Asp, Met, Cys, Gly, Leu, Thr_2, Glu)Lys

Cysteine was identified as *S*-(1,2-dicarboxyethyl)-cysteine formed on hydrolysis of the *N*-ethylmaleimide adduct in a yield of about 30% [10].

3.2. *Reaction with iodo[2-^{14}C]acetic acid in urea and isolation of radioactive peptides*

Alkylation of partly and fully buried SH groups of the aminotransferase was carried out as follows. To 2 ml of the enzyme solution (10 mg/ml) in 0.1 M Tris-HCl buffer, pH 8.0, was added 0.5 ml of the solution of iodo[2-^{14}C]acetic acid (3 μmole) adjusted to pH 8.0, followed by solid urea to a final conc. of 8 M. The reaction mixture was incubated in the dark for 2.5 hr at 37° or for 5 hr at 22°. The reaction was stopped by adding 10 μl of 2-mercaptoethanol; the mixture was heated for 5 min at 90°, then dialyzed at 4° against several changes of 0.001 N HCl.

A portion of the protein solution was freeze-dried and hydrolyzed with 5.7 N HCl (22 hr, 105°). One single radioactive spot was detected on a peptide map of this hydrolysate; the position of the spot coincided with the position of an authentic sample of *S*-carboxymethylcysteine. This fact indicates that no other groups besides SH were alkylated under the above-mentioned conditions.

The ^{14}C-carboxymethylated enzyme was digested with trypsin (1:30, w/w) for 5 hr; the clear solution was then adjusted to pH 5.5 and freeze-dried. The digest was dissolved in 50 mM NH_4OH, applied to paper and subjected to electrophoresis at pH 6.5. A single radioactive band corresponding in its mobility to neutral amino acids was revealed by radioautography, but some radioactive material (probably containing non-mobile "core" peptides) remained at the origin. The "neutral" band was cut out, sewn onto a

* During chromatography a second, much weaker radioactive band was separated; its electrophoretic and chromatographic behaviour was very similar to that of the main band. The composition of this additional band is under investigation.

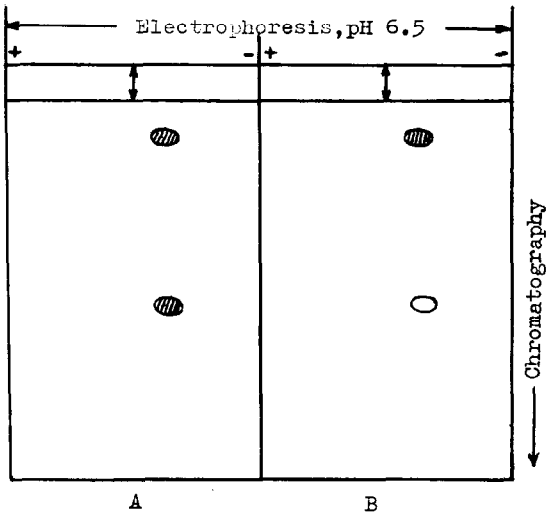

Fig. 1. Radioautographs of tryptic peptide maps of ^{14}C-carboxymethylated aminotransferase. A) Enzyme treated with iodo[2-^{14}C]acetic acid in 8 M urea following alkylation of exposed SH groups with "cold" iodoacetic acid. B) Same as A, but prior to addition of iodo[2-^{14}C]acetic acid the enzyme was syncatalytically alkylated with "cold" *N*-ethylmaleimide, as described in [6]. Intensity of the lower, fast spot was greatly decreased in this experiment; it thus contains the peptide with the functionally important Cys.

fresh sheet of paper and submitted to chromatography; this resulted in separation of two radioactive spots with different chromatographic mobilities (their R_f values were ~0.2 and ~0.5).

Intensity of the faster spot on radioautographs was greatly decreased in experiments where aminotransferase had been syncatalytically alkylated with "cold" *N*-ethylmaleimide prior to the treatment with iodo-[2-^{14}C]acetic acid in urea (see fig. 1). This finding indicated that the fast ^{14}C-labelled spot contained the peptide with the functional cysteine residue. This peptide was further purified by electrophoresis at pH 3.5, rechromatography and electrophoresis at pH 2.0. The N-terminal residue and amino acid composition of the peptide proved identical to those of the peptide (described above) isolated upon syncatalytic alkylation of the enzyme with *N*-ethyl[^{14}C]-maleimide (with the difference that *S*-carboxymethylcysteine was found instead of *S*-(1,2-dicarboxyethyl)-cysteine).

Comparison of our data with the published partial peptide sequence of aspartate aminotransferase [3] indicates that the functionally important cysteine residue is localized in the C-terminal part of the enzyme's polypeptide chain (tryptic fragment No. 22 in [3]). The syncatalytically reactive cysteine residue was independently and simultaneously identified by P. Christen and associates (communication at the 8th FEBS Meeting [11] and who kindly sent a preprint of a forthcoming paper) who used other procedures for labeling its SH group.

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