IDENTIFICATION OF THE EXPOSED AND BURIED CYSTEINE RESIDUES IN THE PEPTIDE SEQUENCE OF ASPARTATE AMINOTRANSFERASE FROM PIG HEART CYTOSOL

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SUMMARY

The position of the two exposed and of one fully buried cysteine residues in the polypeptide chain of aspartate aminotransferase was established. The exposed residues are Cys-45 and Cys-82, the buried one is Cys-252. The functionally important, semiburied cysteine residue of the enzyme was previously found to be Cys-390. Available evidence indicates that the remaining fully buried cysteine residue - the one most difficultly accessible for modification - is Cys-191. Thus, the positions of all five cysteine residues of the aminotransferase molecule are identified.

L-Aspartate:2-oxoglutarate aminotransferase (EC 2.6.1.1; Asp-aminotransferase) from pig heart cytosol consists of two identical subunits of molecular weight about 46,340 (1,2). Each subunit has 5 thiol groups (2-8): two exposed SH groups, readily modified by iodoacetate with no decrease in enzymic activity; two fully buried SH groups inaccessible to thiol reagents in the native enzyme, and one relatively non-reactive, functionally important SH group, probably situated in proximity to the active site. This latter SH group is not accessible to alkylating reagents in the absence of substrates, but can be blocked with p-mercuribenzoate at pH 4,6 (3). In the presence of a substrate pair it is subject

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to "syncatalytic" modification by N-ethylmaleimide or 5,5'-dithiobis(2-nitrobenzoate) with 95% inactivation of the enzyme. Recent findings of Birchmeier et al.(6) and Torchinsky et al. (7) indicate that this SH group belongs to residue Cys-390 of the polypeptide chain (2). The present communication reports identification of the two exposed and one fully buried cysteine residues in Asp--aminotransferase.

MATERIALS AND METHODS

Asp-aminotransferase was purified as previously described (3,9). Protein was estimated spectrophotometrically, taking $A^{1\%}_{1cm}$ = 14.0 at 280 nm. SH groups were determined with p-mercuribenzoate according to Boyer (10) or with the Ellman reagent. Iodo/2-^{14}C/ acetic acid (from the Radiochemical Centre, Amersham) was diluted with carrier iodoacetic acid to a specific radioactivity of 8 mCi/ /mmole. The enzyme protein in 1% NH_4HCO_3 solution, pH 8.0, was digested with trypsin (1:50, w/w) for 22 h at 37°. Bovine trypsin, pretreated with diphenylcarbamylchloride, was a product of PL-Biochemicals, Inc.

Radioactive tryptic peptides were detected on paper by radioautography (using RT-2 X-ray film, exposure 48 h), following separation and purification by means of high-voltage electrophoresis (70 V/cm) and chromatography on Whatman paper 3MM. Descending chromatography was done in the solvent system, pyridine-butan--1-ol-acetic acid-water (10:15:3:12). Peptides were hydrolyzed in 5.7 N HCl (22 h ,105°) in sealed evacuated tubes. Amino acid analyses were done with the Bio-Cal Model 200 analyzer. N-terminal residues and C-terminal amino acids (released with carboxypeptidase A or B) of peptides were identified by the dansyl procedure (11). Amino acid sequences were established by the combined dansyl-Edman procedure (11,12).

RESULTS

Identification of the exposed cysteine residues. Labeling of the exposed cysteine residues of Asp-aminotransferase was carried out as follows. To a solution of native enzyme (8-16 mg/ml) in 0,1 M Tris-HCl buffer, pH 8.0, 2-mercaptoethanol was added in fourfold molar excess. After 1 h five equivalents of neutralized iodo/^{14}C/acetic acid per reactive SH group present in the sample were added, and the solution stayed in the dark at room t° for 22 h. / Under such conditions only the readily accessible SH groups of the enzyme are alkylated (3)/. Excess labeled reagent was then removed by dialysis and the protein was denatured in 8 M urea (3 h, 37°, pH 8) in the presence of "cold" iodoacetate. Urea and iodoacetate were removed by dialysis against 1 mM HCl at 4°. After dialysis the protein was digested with trypsin and freeze-dried. The digest was dissolved in 50 mM NH_4OH, streaked on paper and subjected to electrophoresis at pH 6,5. Radioautography revealed two radioactive bands : one corresponding in mobility to neutral amino acids and another band migrating towards the anode. Both bands were cut out, sewn onto fresh sheets of paper and submitted to chromatography; each band contained one single radioactive peptide (Fig. 1,A). Their R_f values were ~0,19 and ~0,35. The peptides were further purified by electrophoresis at pH 3,5 and rechromatography. The N-and C-terminal residues of the neutral peptide were identified as Thr and Arg, respectively. The amino acid composition of this peptide was (nmoles): Cys(Cm) 17 *, Thr 19, Ser 25, Ala 26, Arg 23. Therefore the peptide may be represented as follows:

Thr/Cys(Cm),Ala,Ser/Arg

* Low recovery of Cys(Cm) is often observed with peptides purified by paper electrophoresis (13).

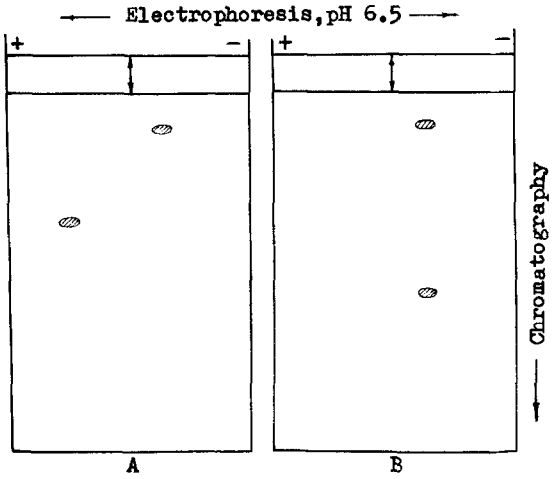

Fig. 1 The position of radioactive peptides on tryptic peptide maps of ^{14}C-carboxymethylated aminotransferase.
A. Peptides with label in the exposed cysteine residues.
B. Peptides with label in the buried cysteine residues.

When compared with the enzyme's primary structure (2) these data allow to conclude that the peptide contains residues 81-85, including Cys-82.

The radioactive peptide with anionic mobility also had Thr and Arg as the N- and C-termini. By three cycles of Edman degradation the following N-terminal partial sequence was established: Thr-Asp-Asp-. Since this peptide was obtained in low yield, its amino acid composition was ascertained only by the dansyl procedure; Cys(Cm), Glu, Pro, Val, Leu were found in addition to the above-mentioned Thr, Asp and Arg. Thus the partial sequence may be represented as follows:

Thr-Asp-Asp/Cys(Cm),Glu,Pro,Val,Leu/Arg

By comparison with the primary structure (2) we conclude that the acidic radioactive peptide containes residues 42-54, including Cys-45.

Identification of the buried cysteine residues. Selective labeling of the buried ("masked") SH groups of the enzyme was carried out in the following way. First the two exposed SH groups in native enzyme were carboxymethylated by incubation with 50 mM "cold" iodoacetate in 0,1 M Tris-HCl buffer (pH 8.0, 24 h ,4°); excess iodoacetate was removed by dialysis. After dialysis the buried SH groups were alkylated with iodo[^{14}C]acetic acid in the presence of 8 M urea; 6 μmoles of neutralized iodo[^{14}C]acetic acid were added per 1 μmole (46.5 mg) of protein and the mixture was incubated for 3 h at pH 8 and 37°. The reaction was stopped by adding an excess of 2-mercaptoethanol; the mixture was heated for 5 min at 90° and dialyzed at 4° against 1 mM HCl. The ^{14}C-carboxymethylated enzyme was then digested with trypsin and freeze-dried. The digest was subjected to paper electrophoresis at pH 6.5. Two radioactive bands were revealed by radioautography: a band corresponding in mobility to neutral amino acids, and a band remaining at the origin (probably containing non-mobile "core" peptide). The neutral band was cut out and submitted to chromatography; this resulted in separation of two radioactive spots with different chromatographic mobilities (Fig. 1,B). Their R_f values were ~0.2 and ~0.5.

The faster-moving spot contains the peptide with functionally important Cys-390, as shown previously by Torchinsky et al. (7). The slower radioactive peptide was purified by electrophoresis at pH 3.5 and rechromatography; it had Tyr as the N-terminal and Lys as C-terminal amino acid. This large peptide was digested with chymotrypsin (4 h, 37°, pH 8), and the digest was fractionated by electrophoresis at pH 6.5. Two new radioactive fragments were revealed; one of these was purified and analyzed. Its N- and C-terminal residues proved Glu and Phe, respectively. Three

cycles of Edman degradation revealed the following N-terminal sequence: Glu-Leu-Phe-. The total amino acid composition of the fragment was as follows (nmoles): Cys(CM)18, Glu 62, Ser 26, Ala 32, Leu 28, Phe 54. Thus the peptide fragment may be represented as:

Glu-Leu-Phe/Cys(CM),Ala,Glu,Ser/Phe

This corresponds to the peptide fragment comprizing resiaues 249-256 in the primary structure of the enzyme and accordingly includes the buried Cys-252. It may be inferred that the remaining profoundly buried cysteine residue is Cys-191. This residue is a component of the large tryptic peptide (2) which probably remains at the origin during paper electrophoresis.

Similar results (concerning the positions of cysteine residues in Asp-aminotransferase) were independently obtained by Polyanovsky et al. (1973, personal communication), who used other procedures for identification of the SH groups.

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