LOCATION OF EXPOSED AND BURIED CYSTEINE RESIDUES IN THE POLYPEPTIDE CHAIN OF ASPARTATE AMINOTRANSFERASE

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1. Introduction

The polypeptide chain of aspartate aminotransferase (Asp-transaminase) from pig heart cytosol contains five cysteine residues [1], which relative to their accessibility for interaction with alkylating agents fall into an exposed and a buried (or screened) category. As demonstrated by several authors, the enzyme molecule (mol. wt. 46 340) possesses two exposed and, consequently, three buried SH groups [2, 3]. In the present work both the exposed and screened thiol groups have been selectively modified by [^{14}C]iodoacetate. The radioactively labeled peptide fragments were isolated and partially sequenced. Identification of the fragment structures with corresponding parts of the enzyme polypeptide chain has demonstrated that the external thiol groups belong to cysteine residues 45 and 82, and the internally situated masked thiol groups to Cys-252 and Cys-390. Residue Cys-191 is likewise masked, its SH group apparently being the most deeply buried of the SH groups in the enzyme protein.

2. Materials and methods

Asp-transaminase was purified as described earlier [4], with slight modifications.

Thiol groups were determined by titration in 1% sodium dodecylsulfate according to Boyer [5] and to Ellman [6]. The exposed thiol groups were carboxymethylated by treating the enzyme at pH 8.5 for 3 hr with [^{14}C]iodoacetate, in a 1:0.7 protein: iodoacetate molar ratio followed by addition of excess cold iodoacetate. After removal of the non-reacted iodoacetate, the protein was denatured (8 M urea, pH 10.5, 24 hr), subjected to dialysis against 0.1 M ammonia and lyophilized.

To label the internal thiol groups of Asp-transaminase the exposed SH groups were first carboxymethylated with cold iodoacetate; after removal of excess iodoacetate the protein was denatured and then alkylated for 3 hr with [^{14}C]iodoacetate in 8 M urea at pH 8.5. The subsequent procedure was the same as indicated above for labeling the exposed SH groups.

Lyophilized labeled protein samples were dissolved in 75% formic acid and subjected to cleavage with a 150–300-fold excess of freshly synthesized CNBr (prepared according to ref. [7]) for 24 hr. The peptide mixture was lyophilized, dissolved in 8 M urea (pH 8.1) and acylated with citraconic anhydride in a TTT-11 (Radiometer, Denmark) titrimeter cell. Tryptic digestion of the peptides was likewise performed in the titrimeter cell (pH 8.3; 37°C).

The peptides were separated by column chromatography on Sephadex, polyacrylamide resins and cellu-

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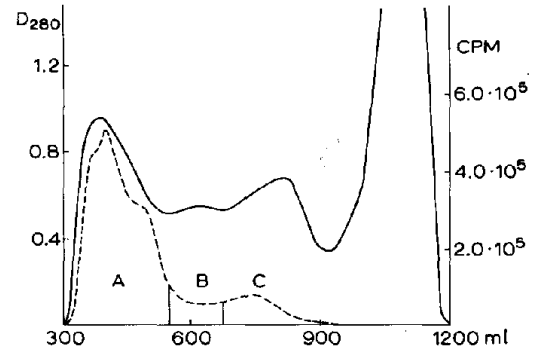

Fig. 1. Separation of peptide mixture obtained by CNBr-cleavage of Asp-transaminase labeled in the 'exposed' thiol groups with [^{14}C]iodoacetate. Fractionation on a Sephadex G-75 (29 × 170 cm) column; 0.1 M ammonia solution in 6 M urea: (——), absorbance at 280 nm; (·····), imp. min^{-1}. ml^{-1}.

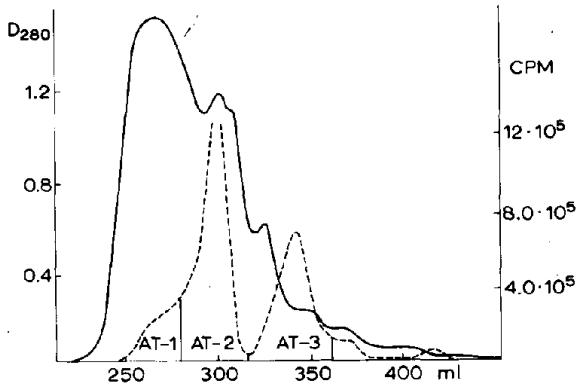

Fig. 2. Separation of fraction A (see fig. 1) on a Sephadex G-25 column in 0.1 M ammonia solution. (Designations as in fig. 1).

lose DE-32 as well as by paper chromatography and electrophoresis using Whatman 3 paper. Fractionation of CNBr and core peptides on Sephadex, Bio-gel and DE-32 columns was performed in 6–7 M urea solutions.

Radioactivity levels were measured in the eluted fractions by means of an SL-30 (Intertechnique, France) scintillation counter; 50 μl samples were added to scintillant in 10 ml of dioxane; in all cases the averages of two scintillation assays were taken.

After purification of each peptide an aliquot was hydrolyzed under standard conditions (5.7 N HCl; 105°C; 24 hr; for valine estimation, 72 hr). The amino acid content was determined in a Bio-Cal (Munich), Model BC201 analyzer. N-terminal residues and sequences were determined by the Edman method as modified by Gray [8].

3. Results

3.1. *Isolation of peptides containing exposed thiol groups*

Asp-transaminase, with the two exposed SH groups alkylated, was cleaved with CNBr and the peptides were fractionated on Sephadex G-75 (fig. 1). The bulk of the radioactivity was recovered in fraction A containing the N-terminal CNBr-fragment (residues 1–212). This fraction after desalting was digested with trypsin, and then chromatographed on Sephadex G-25. As seen in fig. 2 nearly all the radioactivity in the tryptic hydrolysate was localized in two peaks (AT-2 and AT-3). Fraction AT-3, corresponding to a peptide of smaller size, was chromatographed on a Sephadex G-15 column and then purified by paper electrophoresis (30 $V \cdot cm^{-1}$; pyridine acetate, pH 5.6) and paper chromatography in the system pyridine–butanol–acetic acid–water (10:15:3:12). The characteristics (N-terminal residue and amino acid composition) of the purified peptide are presented in table 1. This tryptic peptide (AT-3-1) coincides with the peptide of the limited tryptic digest (residues 81–85; refs. [1, 9]) and, accordingly, includes the Cys-82 residue.

Fraction AT-2, after gel-filtration on a column of Sephadex G-50 and paper chromatography on Whatman 3, yielded a pure peptide, the amino acid composition and N-terminus of which are shown in table 1. This peptide (AT-2-1) corresponds to the fragment of the peptide chain (residues 42–54; refs. [1, 9]), which includes Cys-45. From fraction AT-1 (fig. 2), upon rechromatography on Sephadex G-50 small amounts could be recovered of a peptide containing the Cys-191 residue; the peptide was non-radioactive.

Table 1
Amino acid composition and N-terminal residues of peptide fragments containing cysteine.

	AT-3-1	AT-2-1	CNBr-7	BT-1	ATC-1
Lys	–	–	1.92 2	1.10 1	–
His	–	–	0.90 1	–	1.82 2
Arg	0.94 1	1.10 1	–	0.91 1	–
Trp	–	1	–	–	1
Cys (CM)	0.84 1	0.65 1	0.66 1	0.75 1	0.61 1
Asp	–	2.07 2	2.01 2	1.82 2	1.86 2
Thr	1.02 1	1.25 1	3.60 4	–	3.20 3
Ser	1.15 1	–	1.31 1	2.41 3	–
Glu	–	0.93 1	2.46 2	4.22 4	2.21 2
Pro	–	2.20 2	–	–	2.73 3
Gly	–	–	1.21 1	2.40 2	1.28 1
Ala	1.04 1	–	1.85 2	1.31 1	1.98 2
Val	–	2.60 3	1.73 2	0.91 1	0.89 1
Hse	–	–	–	–	–
Ile	–	–	1.84 2	–	–
Leu	–	0.90 1	1.86 2	1.80 2	0.91 1
Tyr	–	–	0.95 1	1.95 2	–
Phe	–	–	–	4.87 5	–
Total	5	13	23	25	19
N-terminal	Thr	Thr	Cys (CM)	Tyr	Val

The first column gives the analyser data computed per mole for each peptide; the second column is the expected amount of amino acid, based on the complete primary structure of aspartate aminotransferase [1].

3.2. *Isolation of peptides containing 'buried' thiol groups*

The results of fractionating the CNBr-cleaved Asp-transaminase peptides containing the deep-seated thiol groups carboxymethylated by [^{14}C]iodoacetate are shown in fig. 3. Fractions D and C were separated on a Sephadex G-50 column from an admixture of fraction B. The ^{14}C-labeled fractions (C′ and D′) were combined and subjected to column chromatography on DE-32 in 7 M urea, using an NaCl gradient. On elution a labeled peptide was obtained, which had the N-terminal sequence Cys(CM)–Gly–Leu–... and the amino acid composition shown in table 1 (CNBr-7). This peptide corresponds to the C-terminal fragment of the Asp-transaminase peptide chain, comprising residues 390–412. It should be noted that this was the first successful isolation of the C-terminal fragment of CNBr-cleavage of the enzyme. Its purification and identification are facilitated by the specific label on the Cys-390 residue.

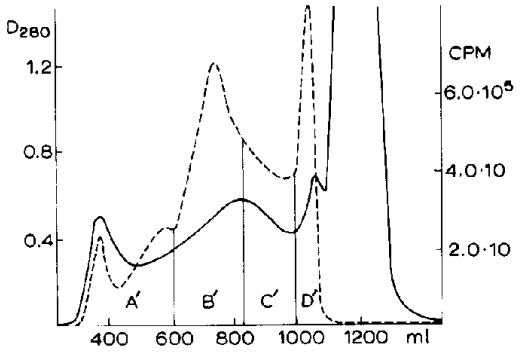

Fig. 3. Separation of peptide mixture obtained by CNBr-cleavage of Asp-transaminase selectively carboxymethylated with [^{14}C]iodoacetate on buried thiol groups after preliminary alkylation of the exposed thiol groups with cold iodoacetate. Fractionation on a Sephadex G-75 column.

Fraction B is a complex aggregate of fragments belonging to the central portion of the enzyme polypeptide chain. This fraction was subjected to tryptic digestion, followed by separation of the hydrolysates on a Bio-gel P-10 column. It was further purified on DE-32 in 6 M urea. The labeled peptide (BT-1) thus obtained had an N-terminal tyrosine residue. The N-terminal sequence of the peptide, determined by the

Table 2
Classification of cysteine residues in the aspartate aminotransferase molecule.

Position in the peptide chain	Characteristics	Category
Cys- 45	1. Alkylatable in undenatured protein without impairment of enzyme activity 2. Selectively adds one maleate residue 3. Is in conformational interaction with the active site	Exposed
Cys- 82	Alkylatable in native enzyme without impairment of catalytic activity.	Exposed
Cys-390	Undergoes selective alkylation or oxidation with marked impairment of catalytic function, in the presence of a substrate pair [11–14]; modifiable on partial denaturation in the absence of substrates	Semi-buried, functionally important
Cys-252	Alkylatable upon preliminary denaturation of the protein	Buried
Cys-191	Modifiable with great difficulty in denaturing media	Most deeply buried

Edman technique, was Tyr–Phe–Val–Ser–...; its amino acid composition is presented in table 1; these characteristics correspond to the 26-membered peptide (residues 241–266), including the Cys-252 residue.

Fraction A (fig. 1), consisting mainly of the 212-membered N-terminal CNBr-fragment was hydrolyzed with trypsin, and the tryptic digest was fractionated on a Bio-gel P-10 column. The 46-membered core peptide (residues 167–212) formed on tryptic digestion had low radioactivity as compared to the Cys-252- and Cys-390-containing peptides.

This core peptide was cleaved with chymotrypsin, and the digest was subjected to chromatography on a Sephadex G-50 column. The amino acid composition of purified radioactive peptide (ATC-1) is shown in table 1.

4. Discussion

Knowledge of the complete primary structure of Asp-transaminase opened the way for locating functionally active amino acid residues in the peptide chain.

In this study the position of exposed and masked cysteine residues was determined; the topological functional classification of cysteine residues in the enzyme's peptide chain is given below. The most readily accessible residue, Cys-45 undergoes alkylation in the absence of denaturing agents; it is the only cysteine residue of the undenatured protein adding to the double bond of maleic acid [10]. It has been shown that Cys-45 is situated in a region of the enzyme molecule that is conformationally sensitive to interactions of the active site with specific ligands [10]. The residue is situated in the neighbourhood of the functionally important Tyr-40 which undergoes nitration on inactivation of the enzyme with tetranitromethane [11, 12]. Despite its presumable vicinity to the active site, modification of Cys-45 does not impair the enzyme's catalytic properties. Comparison of the structures of syncatalytically modified cysteine containing peptides [13, 14] with the complete polypeptide chain of Asp-transaminase allows the functionally important syncatalytically susceptible residue to be identified as Cys-390. This residue is more easily accessible than the following two cysteines, and has been designated as semi-buried [13, 15]. The buried Cys-252 is not very distant from Lys-258; its functions, if any, are unknown. The most deeply buried cysteine is residue 191; it is practically nontitratable in SDS solution and is only with great difficulty accessible to [^{14}C] iodoacetate labeling after denaturation of the protein with 8 M urea. The results of our studies are summarized in table 2.

Zufarova, Torchinsky, Severin, et al. (personal communication, 1973) using a different experimental programme, independently reached concordant conclusions concerning the functional–topological distribution of the cysteine residues in the peptide chain of aspartate aminotransferase.

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