IDENTIFICATION OF CYSTEINE RESIDUES IN ASPARTATE TRANSAMINASE FROM PIG-HEART CYTOSOL

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Aspartate α-ketoglutarate transamines (E.C. 2.6.1.1) from pig-heart cytosol consists of two identical subunits with mol. wt. 46,340, each of which contains five SH groups [1, 2]. These SH groups can be classified in the following way [3-8]:

a) two readily accessible, reacting with iodoacetate without reduction in the activity of the enzyme;

b) two completely masked, accessible to reagents only after the denaturation of the protein; and

c) one partial masked, functionally important, and apparently located in the region of the active center of the enzyme. The last-mentioned group is inaccessible to alkylating reagents in the absence of substrates, but can be blocked by p-mercuribenzoate at pH 4.6 [3]. In the presence of an amino and keto substrate, i.e., during the action of the enzyme, it is subjected to syncatalytic modification of N-ethylmaleimide or 5,5'-dithiobis(2-nitrobenzoate) with 95% suppression of the enzyme activity [5, 6]. We [7], and also Birchmeier et al., [6] have found that this SH group belongs to the cysteine residue occupying position 390 in the peptide chain of the enzyme.

The present paper gives the results of the identification of the readily accessible and the completely masked SH groups of aspartate transaminase from pig-heart cytosol.

The readily accessible cysteine residues were labelled in the native enzyme by alkylation with [^{14}C] iodoacetic acid. Then the protein was digested with trypsin and the hydrolyzate was investigated by paper electrophoresis at pH 6.5. The electrophoretogram showed two radioactive bands: a band coinciding in mobility with neutral amino acids and a band migrating on electrophoresis in the direction towards the anode. Both bands were cut out, stitched to new sheets of paper, and chromatographed. The radioautograms of the peptide map showed that each of the bands contained one radioactive peptide. The R_f values of these peptides were ~0.19 and ~0.35. Then the peptides were purified by electrophoresis at pH 3.5 and by rechromatography. The peptide with neutral properties (less mobile on chromatography) contained threonine at the N end and arginine at the C end. An amino-acid analysis of an acid hydrolyzate of this peptide showed the presence of the following amino acids (μmole): Cys(CM) 0.017, Thr 0.019, Ser 0.025, Ala 0.026, Arg 0.023, and traces of Gly. Thus, the peptide may be shown in the form Thr[Cys(CM), Ala, Ser]Arg.

On comparing the structure of the peptide with the primary structure of the transaminase, it can be seen that we had isolated the peptide 81-85 including the Cys-82 residue.

The peptide with more acidic properties also had threonine at the N end and arginine at the C end. The N-terminal segment of the peptide had the sequence Thr-Asp-Asp. The composition of this peptide was determined by the quantitative dansyl method;* in addition to those already mentioned, the amino acids Cys(CM), Glu, Pro, Val, and Leu were found. Consequently, the peptide can be represented in the form: Thr-Asp-Asp[Cys(CM), Glu, Pro, Val, Leu]Arg. On comparing its composition with the primary structure of the enzyme (see [2]), it may be concluded that this peptide contains the Cys-45 residue. The masked

*The peptide was obtained in low yield and was therefore not subjected to quantitative amino-acid analysis.

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cysteine residues were labelled selectively. On peptide maps of an acid hydrolyzate, only one radioactive spot was found, which coincided in electrophoretic and chromatographic mobilities with a standard sample of carboxymethylcysteine. It follows from this that under the conditions of our experiments the [^{14}C] iodoacetic acid reacted only with the cysteine residues in the protein.

The carboxymethylated enzyme was digested with trypsin and the digestion products were separated by paper electrophoresis at pH 6.5. The electrophoretogram showed two radioactive bands: a band coinciding in mobility with the neutral amino acids and a band remaining at the position of deposition and obviously belonging to a large "core" (heart) peptide. The neutral radioactive band was cut out, stitched to a new sheet of paper, and chromatographed, after which two radioactive spots separated. The spots differed considerably in chromatographic mobility (R_f values 0.2 and ~0.5). The more mobile spot contained, as we have established previously, a peptide with the functionally important Cys-390 residue [7]. The radioactive peptide present in the less mobile spot was purified by electrophoresis at pH 3.5 and was chromatographed. It was found that its N-terminal residue was tyrosine and its C-terminal residue lysine. Then the peptide was digested with chymotrypsin (4 H, 37°C, pH 8). Two new radioactive fragments were produced, one of which (obtained in higher yield) was purified and analyzed. The N-terminal residue in it proved to be glutamic acid and the C-terminal residue phenylalanine. The sequence Glu-Leu-Phe was found in the N-terminal segment of this peptide fragment. An amino-acid analysis of an acid hydrolyzate showed the following amino acids (μmole): Cys(CM) 0.018,* Glu 0.062, Ser 0.026, Ala 0.032, Leu 0.028, Phe 0.054. Consequently, the peptide fragment found can be represented in the form Glu-Leu-Phe [Cys(CM), Ala, Glu, Ser] Phe.

The results of a comparison of the structure of this fragment with the primary structure of the enzyme (see [2]) permitted the conclusion that it contained the Cys-252 residue.

Thus, in addition to the previously identified functionally important cysteine residue (Cys-390) we have also identified the two readily accessible residues (Cys-45 and Cys-82) and one completely masked cysteine residue (Cys-252). It may be assumed that the second completely masked cysteine residue (obviously Cys-191) is present in the large "core" peptide which does not move from the start in paper electrophoresis.

EXPERIMENTAL

Materials. The enzyme was purified and its activity was measured as described previously [3, 9]. The number of SH groups was determined by titration with p-mercuribenzoate by Boyer's method [10] and also by means of Ellman's reagent. Trypsin was used which had been treated with diphenylcarbamoyl chloride (PL-Biochemicals, Inc.), chymotrypsin (A grade) and carboxypeptidases A and B (Calbiochem), and [2-^{14}C] iodoacetic acid (Radiochemical Center, Amersham, England), which was diluted with "cold" iodoacetic acid to a specific radioactivity of 8 mCi per mmole.

Digestion of the Protein with Trypsin. The process was performed in 1% NH_4HCO_3 solution (pH 8, 37°C, 22 h); the trypsin was added in two portions, each being 1/50 of the weight of the transaminase. The peptides were purified by electrophoresis and were chromatographed on Whatman 3 MM paper. The radioactive peptides were revealed on the paper by radioautography on RT-2 x-ray film (exposure time 48 h). Electrophoresis was performed on a sheet of paper arranged vertically and immersed in a chamber with the cooling liquid (white spirit) at a voltage of 70 V/cm at pH values of 6.5 and 3.5. Chromatography was performed in the pyridine–butan-1-ol–acetic acid–water (10 : 15 : 3 : 12) system. The peptides were hydrolyzed in 5.7 N HCl (105°C, 22 h) in tubes sealed under vacuum. The amino-acid compositions of the peptides were determined on Bio-Cal model BC 200 and Beckman Unichrom analyzers.

The N- and C-terminal amino-acid residues were found by the dansyl method [11] and the C-terminal residues after cleavage with carboxypeptidase A or B. Incubation with the carboxypeptidases was performed in 0.2 M N-ethylmorpholine acetate buffer (pH 8.5, 37°C, 2 h); the enzyme was added in an amount of 10 γ per ~0.02 μmole of peptide. The sequences of the residues in the peptides were determined by a micro modification of Edman's method in combination with dansylation [11, 12]. The dansylamino acids were identified by two-dimensional chromatography on plates with a thin layer of type KSK silica gel.

Identification of the Readily Accessible Cysteine Residues. 2-Mercaptoethanol in a twofold excess with respect to the number of readily accessible SH groups of the protein was added to a solution of the en-

*The low yield of carboxymethylcysteine is a general phenomenon in the purification of peptides on paper [13].

zyme in 0.1 M tris-HCl buffer, pH 8.0; the mixture was incubated for 1 h, after which neutralized [^{14}C]iodoacetic acid was added to it in fivefold excess on the total number of free SH groups. The final concentration of [^{14}C]iodoacetic acid was 4-8 mM and the concentration of protein 8-16 mg/ml. The mixture was incubated at room temperature in the dark for 22 h. Under these conditions only the readily accessible SH groups are alkylated [3]. The completeness of the alkylation reaction was followed by means of Ellman's reagent. Then the solution of the enzyme was dialyzed to eliminate the [^{14}C]iodoacetic acid that had not reacted, after which the protein was denatured in 8 M urea (pH 8, 37°C, 3 h) in the presence of an excess of "cold" iodoacetate. The urea and the iodoacetate were eliminated by dialysis against 0.001 N HCl at 4°C, and the protein was digested with trypsin and freeze-dried. The dry residue was dissolved in 0.05 N NH_4OH and deposited on paper for the performance of electrophoresis at pH 6.5.

Identification of the Masked Cysteine Residues. First the readily accessible SH groups were blocked by incubating the native enzyme with 0.05 M nonradioactive iodoacetate (pH 8, 4°C, 24 h). The excess of iodoacetate was eliminated by dialysis, and then the masked SH groups were labelled by alkylation with [^{14}C]iodoacetic acid in the presence of 8 M urea. Neutralized [^{14}C]iodoacetic acid was added in an amount of 6 μmole per μmole (46.5 mg) of protein. The mixture was incubated in the dark (pH 8, 37°C, 3 h). Then to it was added an excess of 2-mercaptoethanol and it was heated at 90°C for 5 min. The reagents were eliminated by dialysis against 0.001 N HCl at 4°C. After dialysis, part of the protein solution was freeze-dried and hydrolyzed in 5.7 N HCl (105°C, 22 h).

SUMMARY

The two readily accessible cysteine residues (Cys-45 and Cys-82) and one completely masked residue (Cys-252) in the polypeptide chain of aspartate transaminase have been identified. The second completely masked cysteine residue is Cys-191.

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