IDENTIFICATION OF THE REACTIVE SULPHYDRYL GROUP OF MITOCHONDRIAL ASPARTATE AMINOTRANSFERASE FROM PIG HEART

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1. Introduction

The cysteine residues of cytoplasmic aspartate aminotransferase (s-AAT) from pig heart have been extensively investigated [1–6]. In the native protein, the five sulphydryl groups per polypeptide chain fall into three classes on the basis of their reactivities. Two residues (at positions 45 and 82 in the sequence [7,8]) react rapidly with a variety of sylphydryl reagents, the modification causing little or no change in catalytic activity. Two further residues (positions 191 and 252) appear to be deeply buried and do not react under non-denaturing conditions. The remaining residue (position 390) reacts with 5,5′-dithiobis-(2-nitrobenzoate) (DTNB) approximately 10^5 times more slowly than do residues 45 and 82, but the rate of modification is increased up to 100 times in the presence of enzyme substrates or substrate analogues; this phenomenon has been termed 'syncatalytic modification' [9].

Mitochondrial aspartate aminotransferase (m-AAT) from pig heart contains seven cysteine residues per polypeptide chain [10,11]. Gehring and Christen [11] have shown that only one of these residues is accessible for reaction with DTNB in the native enzyme and that the rate of reaction is increased by the presence of substrates. In view of the established homology of the two isozymes [12] it seemed to be of interest to identify the unique freely-accessible cysteine residue in m-AAT. We report here the amino acid sequence of a peptide containing a cysteine residue labelled by incubation of the native enzyme with [^{14}C]iodoacetic acid in the presence of substrates. The cysteine residue occurs at a position corresponding to residue 80 in the structure of s-AAT.

2. Materials and methods

m-AAT was purified as previously described [10]. Unlabelled iodoacetic acid was recrystallised from toluene; [^{14}C]iodoacetic acid was obtained from the Radiochemical Centre, Amersham, Bucks, UK. *A. mellea* protease was a generous gift from Dr P. L. Walton.

For modification, m-AAT (250 mg, ca. 5.4 μmol) was dissolved in sodium phosphate buffer (0.05 M, pH 7.5, 50 ml) containing pyridoxal-5′-phosphate (0.2 mM), 2-oxoglutaric acid (4 mM) and L-glutamic acid (55 mM) [11]. [^{14}C]iodoacetic acid (4 μmol, 50 μCi) was added followed after 3 h by mercaptoethanol (10 μmol); the solution was left at 4°C overnight. During modification, the majority of the protein precipitated. This precipitate was collected by centrifugation and washed several times with water. The protein was then dissolved in Tris–HCl buffer (0.2 M, pH 8.0, 25 ml) containing urea (8 M), EDTA (0.02 M) and mercaptoethanol (0.586 M). After allowing to stand for 20 h under N_2, unlabelled iodoacetic acid (2.5 g) was added and the solution maintained in the dark for 15 min. The fully-modified protein was then recovered by extensive dialysis against water and finally lyophilized.

For digestion with *A. mellea* protease [7] the modified protein was suspended in *N*-ethylmorpholine–HCl buffer (0.2 M, pH 7.5, 25 ml) and the

protease (2 mg) added in two equal portions, the second 6 h after the first addition. The total time of digestion was 24 h. Acetic acid was then added to 30% by vol and the soluble part of the digest fractionated by passage through a column (2.5 × 42 cm) of Sephadex G-50 equilibrated in acetic acid (30% by vol). Further purification of peptides was carried out as described in the following section.

3. Results

Approximately 30% of the added [^{14}C]iodoacetic acid was incorporated into m-AAT. Of this, 35% was present in the insoluble fraction and the material totally excluded by Sephadex; this presumably represented partially digested or undigested protein. The remainder was approximately equally divided between two peptide fractions designated P2 and P3 in order of elution from the column.

The peptides in fraction P3 were subjected to high-voltage paper electrophoresis at pH 3.5 (Whatman 3 MM paper, buffer system pyridine–acetic acid–water, 1:10:189 by vol, 3 kV, 1 h). Radioactivity was detected only in the slowest-moving cathodal band (using a Tracerlab 4 π Scanner). The radioactive band was eluted (acetic acid, 5% by vol) and the material further fractionated by paper chromatography using the solvent system butan-1-ol–pyridine–acetic acid–water (15:10:3:12 by vol). Radioactivity was detected in the most-rapidly migrating peptide and to a lesser extent in the adjacent band; the activities were in the ratio 3:1.

The major component was subjected to sequence analysis by the dansyl Edman method [13]. The partial sequence found was Lys–Glx–Tyr–Leu–Pro–Ile–Gly–Gly–Leu–Ala–. After residue ten, spots were too weak for reliable identification. Quantitative amino acid analysis showed that in addition to the residues sequenced directly, the peptide contained one residue each of carboxymethyl cysteine and phenylalanine and a further residue of glutamic acid.

A sample of peptide (50 nmol) was subjected to hydrolysis by carboxypeptidase C (Boehringer, Mannheim). The peptide was dissolved in sodium citrate buffer (100 μl, pH 5.3) and digested with protease (0.1 mg) for 120 min at room temperature. Quantitative amino acid analysis showed equal amounts of alanine, carboxymethyl cysteine, glutamic acid, leucine and phenylalanine and a trace of glycine. The results, together with the partial sequence given above, suggest that the C-terminal sequence was –Gly–Leu–Ala– (CMCys, Glu, Phe).

In independent work (D. Barra and F. Bossa, unpublished; see also [12]), the following two overlapping peptides have been isolated after digestion of m-AAT with chymotrypsin and thermolysin respectively:

Asn–Leu–Asp–Lys–Glu–Tyr–Leu–Pro–Ile–Gly–Gly–Leu–Ala–Glu–Phe

and Leu–Ala–Glu–Phe–Cys–Lys–Ala–Ser–Ala–Glu.

It is clear from a comparison of these sequences and of the results given above that the complete structure of the radioactive peptide was Lys–Glu–Tyr–Leu–Pro–Ile–Gly–Gly–Leu–Ala–Glu–Phe–CMCys.

This is also consistent with the known specificity of *A. mellea* protease [7].

The minor radioactive component isolated from fraction P3 by paper chromatography appeared to have the same amino acid sequence and may have differed from the major component by partial oxidation of the carboxymethyl cysteine residue.

From measurements of the specific radioactivity of the major peptide described above it could be calculated that at least 50% of the incorporated radioactivity was located at this cysteine residue. This may be an underestimate since quenching effects during counting were not corrected for. The fate of the remainder of the activity, most of which was associated with peptides from fraction P2, was obviously of interest. Investigations with fraction P2, which will not be described in detail, showed that the radioactivity in this fraction was associated with at least three, and possibly more, different peptides. There was not enough material available to fully characterize these components, but the important point is that the radioactivity not accounted for in the major peptide was incorporated at several different sites in the protein molecule rather than at a single site.

4. Discussion

The results reported by Gehring and Christen [11] show clearly that only one cysteine residue of native m-AAT is accessible to reaction with DTNB. We have demonstrated that with iodoacetic acid also there is one major site of attack and have identified the residue involved. Although the reagent used was different from that employed by Gehring and Christen [11] there seems to be no reason to suppose that the site of reaction will be different in the two cases. In support of this view, the order of reactivities of the cysteine residues of s-AAT is the same with a variety of reagents [1] including iodoacetic acid [3,5,6].

The reactive cysteine residue was located in a peptide which is clearly homologous with residues 68–80 of s-AAT [7]. The sequences are as follows with the numbering referring to the cytoplasmic isozyme:

s-AAT His–Glu–Tyr–Leu–Pro–Ile–Leu–
68

Gly–Leu–Ala–Glu–Phe–Arg–Thr–Cys
82

m-AAT Lys–Glu–Tyr–Leu–Pro–Ile–Gly–

Gly–Leu–Ala–Glu–Phe–Cys

It can be seen that the reactive syncatalytically modifiable cysteine residue of m-AAT is in a position similar to, but not identical with, cysteine-82 of s-AAT whereas the syncatalytically activated cysteine of the latter isozyme is at position 390; it is not yet known whether a cysteine residue occurs at this position in m-AAT.

The difference in location of the syncatalytically reactive groups may perhaps account for some of the obvious differences in the details of modification in the two cases [1,11]. These differences include:
(a) Blocking of the groups with DTNB leads to loss of activity with s-AAT but not with m-AAT.
(b) The syncatalytic activation with m-AAT is of the order of ten fold but approaches 100 fold with s-AAT.
(c) The absolute rates of reaction in the absence of substrates differ by 1000-fold in the two cases. The rate of reaction of the reactive cysteine in m-AAT is closer to those of cysteines-45 and 82 than to that of cysteine-390 in s-AAT.

Hence in some respects, the behaviour of the syncatalytically activated group in m-AAT resembles that of cysteine-82 of s-AAT rather that that of cysteine-390, in agreement with the assignment of the position of the reactive group in m-AAT made in the present work; it should be noted that in m-AAT there is no cysteine residue at the position corresponding to residue 45 of s-AAT [14]. The basic difference remains, however, that the reactivity of cysteine-82 in s-AAT is not increased during the catalytic process. It seems, therefore, that in m-AAT either this region of the polypeptide chain is closer to the active site than is the corresponding region around cysteine-82 in s-AAT, or that the structural change accompanying catalysis extends over a greater distance in the case of m-AAT. It will be interesting to see if m-AAT contains a cysteine residue at the position corresponding to residue-390 in the cytoplasmic isozyme.

Finally, the lack of complete specificity of attack of iodoacetic acid on m-AAT should be noted and contrasted with the behaviour of DTNB. As was pointed out earlier, it appeared that limited reaction occurred at several other points in the polypeptide chain in addition to the major point of attack. This may have been due to reaction at residues other than cysteine (e.g. histidine), which would not have occurred with DTNB, or alternatively reaction of iodoacetic acid with the reactive cysteine residue may cause a structural reorganization of the molecule leading to exposure of other cysteine residues to attack; an effect of the latter type would perhaps account for the observed precipitation of the enzyme during modification.

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