Different Reactivity of Mitochondrial and Cytoplasmic Aspartate Aminotransferases toward an Affinity Labeling Reagent Analog of the Coenzyme*

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The two isoenzymes of aspartate aminotransferase from pig heart have been reacted with a derivative of the coenzyme, 4'-N-(2,4-dinitro-5-fluorophenyl) pyridoxamine-5'-phosphate, which is a potential affinity labeling reagent. The derivative has a great affinity for both isoproteins. In the cytosolic isoenzyme, the reversible binding is followed by a covalent labeling of the α-amino group of lysine 258, which usually forms an aldime bond with pyridoxal-5'-phosphate. In the mitochondrial isoenzyme, no labeling occurs at the active site.

The different reactivity indicates that a small but definite difference exists in the geometry of the two active sites.

In the cytosolic isoenzyme also a sulfhydryl group outside the active site region, namely cysteine 45, reacts, but not by an affinity labeling mechanism.

In both isoenzymes, the reversibly bound reagent slowly undergoes a splitting reaction by which pyridoxal-5'-phosphate is regenerated and activity re-established; the rate of this reaction is not fast enough to impair the labeling potential of the reagent.

The cytoplasmic and mitochondrial isoenzymes of aspartate aminotransferase (EC 2.6.1.1.) from pig heart have been the object of extensive structural research in several laboratories. Although their tertiary structures have not yet been completely unraveled, many features of their surface and active site composition are known. A number of differences between the two proteins have been revealed (1–6). Nevertheless, the active sites of the two proteins show a great similarity in their structural features and in particular in their behavior toward affinity labeling pseudosubstrates (7–11).

In order to gain a deeper insight in the active site structure of the two isoenzymes, both have been tested with an active site-directed reagent which is a derivative of the coenzyme. This reagent is 4'-N-(2,4-dinitro-5-fluorophenyl)-pyridoxamine-5'-phosphate (FP), which has already been shown (12) to be a strong inhibitor of several pyridoxal-5'-phosphate (pyridoxal-P)-dependent apoenzymes and a good reagent for lysyl, cysteinyl, and tyrosyl amino acid side chains (13).

Previous studies (14) have also proved FP to be an affinity labeling reagent toward cytoplasmic aspartase apoenzyme.

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† The abbreviations used are: FP, 4'-N-(2,4-dinitro-5-fluorophenyl)-pyridoxamine-5'-phosphate; pyridoxal-P, pyridoxal-5'-phosphate.

ferase but not toward mitochondrial aspartate apoaminotransferase.

It has, therefore, seemed interesting to further investigate the reaction of both isoenzymes with this inhibitor.

EXPERIMENTAL PROCEDURES

FP was synthesized as previously reported (13); all preparations were checked to exclude pyridoxal-P contamination. Cytoplasmic aspartate aminotransferase from pig heart was prepared according to Martinez-Carrion et al. (15). Mitochondrial aspartate aminotransferase from pig heart was prepared according to Barra et al. (16). The apoenzymes were obtained by a modification (17) of the method of Scardi et al. (18).

Protein concentration was calculated from the optical density at 280 nm 

Carboxymethylation and digestion of the enzymes, purification of peptides by gel filtration, paper chromatography and high voltage electrophoresis, determination of amino acid composition of peptides, and N-terminal analysis were performed according to Riva et al. (21) and Barra et al. (22).

Ion exchange chromatography of peptides was performed on a sulfopropyl (SP)-Sephadex C-25 column (23 × 1.5 cm) at 28°C, eluted with a gradient obtained by placing 250 ml of pyridine (0.2 M)/acetic acid, pH 3.1, in the mixing chamber and 250 ml of pyridine (2 M)/acetic acid, pH 5.1, in the reservoir.

Analytical and preparative chromatography of peptides was performed on cellulose thin layer in butanol/pyridine/acetic acid/water (45:30:9:36).

Carbamylated aspartate aminotransferase was prepared according to Glebe and Martinez-Carrion (23). The modified enzyme is presumably 90% carbamylated since 10% of the original activity can be recovered upon incubation with pyridoxal-P. To block the residual active site lysyl amino groups, the carbamylated apoenzyme was mixed with 10⁻⁴ M pyridoxamine-5'-phosphate and dialyzed in turn against the following solutions: 10⁻² M α-ketoglutarate, 0.05 M Tris-HCl buffer, pH 8, 2 × 10⁻³ M sodium borohydride, and 0.05 M Tris-HCl buffer, pH 8. By this treatment, the residual activity was completely abolished.

Reduced holoenzyme was obtained by treating native holoenzyme with pyridoxamine-5'-phosphate, α-ketoglutarate, and sodium borohydride as described above.

N-Ethylmaleimide-treated apoenzyme, i.e. native apoenzyme in which the 2 more reactive cysteinyl residues were reacted with N-ethylmaleimide, was prepared according to Birchmeier et al. (24).

The reaction of apoenzymes with FP was performed in 0.05 M Tris-HCl buffer, pH 8, in the dark, at room temperature, and was followed by monitoring spectral variations and loss of catalytic activity.
zyme was used in concentration of $2 \times 10^{-3}$ to $2 \times 10^{-4}$m and the labeling reagent was added in slight molar excess. To assess the irreversibility of the binding, the reacted protein was denatured in 0.5 M perchloric acid, a treatment known to remove the coenzyme or noncovalently bound ligands. The precipitate was dissolved in 1 M sodium hydroxide and the spectrum of the solution was analyzed. The evaluation of bound reagent was made spectrophotometrically, assuming an isosbestic point of 405 nm, with $e_{405} = 7,400$ for all of the adducts (13), while the progress of the reaction was followed at 350 nm, where the major variations of absorbance occur.

RESULTS

Reaction with Native Cytoplasmic Aspartate Aminotransferase—The reaction of FP with the cytoplasmic apoenzyme inhibits the recovery of enzymatic activity by addition of pyridoxal-P. When the cytoplasmic apoenzyme was incubated for a few minutes with FP, in 1:1.2 molar ratio, only 10% or less of the original activity could be recovered.

FP, being a phosphopyridoxyl derivative, exhibits high affinity toward aspartate apoenaminotransferase (25) and this fast inactivation might represent mainly a reversible inhibition. In order to measure only the irreversible inhibition, samples of the reaction mixture at different times were incubated at low pH and high ionic strength in the presence of excess pyridoxal-P and the activity was measured again (see "Experimental Procedures"). The inhibition revealed under these conditions reached a maximum of 85% in 8 h (Fig. 1, inset).

The failure to obtain 100% irreversible inhibition is explained by a slow splitting of the reversibly bound reagent, which regenerates pyridoxal-P as revealed by activity measurements without addition of coenzyme. Therefore, the irreversible labeling and the enzyme reactivation are two competing reactions, with a rate ratio of approximately 5:1 in these experimental conditions.

Absorption spectra of the reaction mixture recorded at different times showed the variations reported in Fig. 1. When both the activity and the spectral changes are expressed as per cent of the maximum change (24 h), they follow the same time course. The two phenomena seem, therefore, to be closely correlated (Fig. 2).

![Fig. 1](image-url)

**Fig. 1.** Spectral variations recorded during the reaction of the cytoplasmic apoenzyme with FP. Spectra of apoenzyme (——) and of apoenzyme plus FP after 2 min (— - - -), 150 min (-----), and 24 h (------) of reaction. The reaction of $2 \times 10^{-3}$M native cytoplasmic apoenzyme with $2.4 \times 10^{-4}$M FP was performed in 0.05 M Tris buffer, pH 8, as described under "Experimental Procedures." Inset, per cent inhibition of catalytic activity measured on aliquots of the same reaction mixture after 6 h incubation in 1 M phosphate buffer, pH 5.3, and $5 \times 10^{-4}$M pyridoxal-P.

![Fig. 2](image-url)

**Fig. 2.** Correlation between labeling and inactivation in the binding of the inhibitor to the cytoplasmic apoenzyme. Reaction conditions were the same as in Fig. 1. Absorbance and inhibition values are expressed as per cent of the maximum change (24 h). Open symbols, per cent inhibition of catalytic activity measured as in Fig. 1. Closed symbols, per cent absorbance at 350 nm of the reacted protein after precipitation in 0.5 M perchloric acid of aliquots of the reaction mixture and redissolution of the precipitate in 1 M sodium hydroxide. □ and ▲, native apoenzyme; Δ and A, N-ethylmaleimide-treated apoenzyme.

![Fig. 3](image-url)

**Fig. 3 (left).** Elution profile of the yellow chymotryptic peptides derived from FP-treated cytoplasmic apoenzyme. Chromatography was performed on a SP-Sephadex C-25 column (see "Experimental Procedures"). Collected volumes: 5 ml/tube. Broken line refers to rechromatography of Fraction A after treatment with phosphatase.

**Fig. 4 (right).** Absorption spectra of yellow peptides A, B, and C eluted from SP-Sephadex C-25 (see Fig. 3).

1.05 mol of reagent are incorporated/mol of monomer after 24 h of reaction.

Isolation and Analysis of Labeled Peptides—To identify the labeled residue or residues, the protein was carboxymethylated and cleaved by treatment with chymotrypsin. The peptides were fractionated by chromatography on Sephadex G-25 in 10% acetic acid; only the yellow eluate was collected, the absorption at 350 nm providing an easy way of monitoring the labeled fractions.

From analytical thin layer chromatography, the material absorbing at 350 nm appears to contain three components. Further purification was obtained by ion exchange chromatography on SP-Sephadex (see "Experimental Procedures"). Three yellow elution peaks were obtained, indicated as A, B, and C in Fig. 3.

The spectral features of these fractions (Fig. 4) suggest, by analogy with model compounds (13), the presence of labeled
lysol groups in A and B and labeled cysteinyl groups in C.

Each pooled fraction was treated with alkaline phosphatase according to Strausbauch and Fischer (26) and separately chromatographed on the same SP-Sephadex column under the same conditions. While A was eluted at a less acidic pH (Fig. 3), B and C did not change their elution position.

Fractions A and C were further purified by thin layer chromatography. The NH₂-terminal residues and the amino acid compositions of the yellow peptides are reported in Table I (Columns I and II).

Considering the known sequence of the enzyme (2, 22), the two peptides can be unequivocally attributed to the peptide containing lysine 258 (A) and to the peptide containing cysteine 45 (C).

Analytical thin layer chromatography was performed also on Fraction B. Only one yellow spot was present. Spectral features, R₅ on thin layer cellulose, and elution position upon chromatography on SP-Sephadex were identical for B and the dephosphorylated A, so that B appears to represent a dephosphorylated form of A, probably arising during the purification treatment in acetic acid. The ratio of (A + B) to C was 4:1.

Reaction with Cytoplasmic Apoenzyme in Which Cysteine 45 and 82 Are Blocked by N-Ethylmaleimide—To study lysyl and cysteinyl modifications independently, the reaction with

| Table I |
| --- |
| **Amino acid composition of yellow peptides from labeled apoenzyme and holo cytoplasmic enzyme** |

| Peptide | I | II | III | IV |
| --- | --- | --- | --- | --- |
| Chromotryptic peptide A from 1.8 × 10⁻⁴ M cytoplasmic apoenzyme | **Lys** | 0.7 (1) | 0.9 (1) | 1.1 (1) |
| | **Arg** | 0.5 (1) | 0.6 (1) | 0.8 (1) |
| | **Trp** | 1.0 (1) | 2.0 (2) | 2.0 (2) |
| | **Asp** | 0.9 (1) | 0.8 (1) | 0.8 (1) |
| | **Ser** | 0.8 (1) | 1.0 (1) | 1.0 (1) |
| | **Glu** | 1.0 (1) | 1.0 (1) | 1.0 (1) |
| | **Pro** | 1.0 (1) | 0.9 (1) | 0.8 (1) |
| | ** ε-Cys** | 1.0 (1) | 1.0 (1) | 1.0 (1) |
| | **Val** | 0.9 (1) | 0.8 (1) |
| | **Leu** | 0.8 (1) | 0.8 (1) |
| | **Tax** | 0.9 (1) | 0.8 (1) |
| | **Phe** | 0.8 (1) |

**NH₂-terminal** Ser (Ser) Arg (Arg) Arg (Arg) Tyr (Tyr)

* The modified cysteine could not be identified due to its instability to hydrolysis procedures. Evidence for the presence of cysteine is given in the text.
* Not determined.
* Determined by dansylation.

FP was performed on an apoenzyme in which the more reactive —SH groups had been blocked (see "Experimental Procedures").

The values of inhibition were comparable to those obtained in native cytoplasmic apoenzyme, with a covalent inhibition of 82% after 24 h and a spontaneous reactivation of 18%.

The progress curve of the reaction is shown in Fig. 2 in comparison with native aspartate aminotransferase. 0.8 mol of reagent was incorporated/mol of protein after 24 h of reaction.

Reaction with Cytoplasmic Holoenzyme—To further analyze the labeling reaction, the cytoplasmic holoenzyme was treated with FP in the conditions already described for the apoenzyme.

No effect was noticed on the catalytic activity although FP appears to bind to the protein, as shown by the spectrum obtained after removal of unbound reagent by gel filtration (Fig. 5).

The peptides obtained by chymotryptic digestion were purified by the technique already described for the apoenzyme. The amino acid composition of the main yellow fraction (70% of the yellow material) is reported in Table I (Column III) and corresponds to the peptide containing cysteine 45.

Another peptide, representing less than 30% of the total yellow material, was found. Its spectrum is consistent with a labeled lysine, and its amino acid composition corresponds to the active site peptide. Its presence is puzzling. It might derive from the presence of apoenzyme, from artifacts arising during digestion procedures, or from other unexplained reactions. When the labeling was performed on reduced holoenzyme (see "Experimental Procedures"), no evidence of peptides different from that containing cysteine 45 was found.

As a sensitive measure of the formation of a covalent adduct, the ratio of the absorbance at 350 nm (where the maximum change is noticed when the fluorodinitrophenyl group reacts with a nucleophile) to the absorbance at 280 nm
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which is not significantly affected by the reaction, as shown in Fig. 1) was studied as a function of time after perchloric acid precipitation. The kinetics of the reaction is shown in Fig. 6.

Reaction with Carbamylated Cytoplasmic Apoenzyme—The effect of FP was studied on the apoenzyme which can still bind the coenzyme or its analogs, although the amino group of lysine 258 is made unavailable by carbamylation according to Slebe and Martinez-Carrion (23) (see "Experimental Procedures").

The reaction was performed in the same conditions already described for native cytoplasmic apoenzyme and since the carbamylated apoenzyme is inactive, it was monitored only by following spectral variations.

Fig. 5 shows the spectrum of the protein after 24 h of reaction and removal of unbound reagent by gel filtration.

Peptidases were obtained by peptic digestion; the NH₂-terminal residue and the amino acid composition of the labeled one correspond to the peptide containing cysteine 45 (see Table I, Column IV).

The progress curve of the reaction is shown in Fig. 6, in comparison with the holoenzyme and with the apoenzyme in which the more reactive —SH groups have been blocked.

It can be seen that when the inhibitor in slight molar excess over the enzyme is reacted with the carbamylated apoenzyme, the amount of labeling is lower than in the case of the holoenzyme.

This can be explained by the fact that in the carbamylated apoenzyme the inhibitor binds at the active site and, although it cannot react with the blocked lysine 258, it is unavailable for the reaction with a group which is not near the active site, as cysteine 45.

Reaction with Native Mitochondrial Apoenzyme—The reaction of FP with mitochondrial apoenzyme inhibits the recovery of enzymatic activity by addition of pyridoxal-P and generates spectral changes (Fig. 7A).

No irreversible inhibition follows, as shown by the data reported in Table II, and the competitive inhibition consequent to the large affinity of FP toward the mitochondrial apoenzyme is completely removed by incubation with the coenzyme at pH 8 in Tris buffer.

![Fig. 6. Time course of FP reaction with different forms of the cytoplasmic isoenzyme. Ratio of 350-nm to 280-nm absorbance at different reaction times after precipitation in 0.5 M perchloric acid and redissolution of the precipitate in sodium hydroxide. Reaction conditions were the same as in Fig. 1. , N-ethylmaleimide-treated apoenzyme; , reduced holoenzyme; , carbamylated apoenzyme.](image)

**Fig. 7. Reaction of FP with native mitochondrial apoenzyme.** A, spectral variations recorded during the reaction of mitochondrial apoenzyme with FP. The reaction of 1.9 × 10⁻⁵ M mitochondrial apoenzyme with 2.9 × 10⁻⁵ M FP was performed in 0.05 M Tris buffer, pH 8. The unusual excess of reagent used in this experiment was used to emphasize any possibility of reaction. Spectra of apoenzyme (-----) and of apoenzyme plus FP after 2 min (-----), 150 min (-----), and 24 h (-----) of reaction. B, absorption spectra of mitochondrial and cytoplasmic apoenzymes after 24 h of reaction. The apoenzymes, after perchloric acid precipitation, were dissolved in 1 M sodium hydroxide to the same final concentration. -- X, 8 × 10⁻⁴ M mitochondrial apoenzyme reacted with 10⁻⁵ M FP; ---, 1.6 × 10⁻⁴ M cytoplasmic apoenzyme reacted with 1.9 × 10⁻³ M FP.

**Table II** Variations in the catalytic activity of the mitochondrial apoenzyme during the reaction with FP

Mitochondrial apoenzyme (8.10⁻⁵ M) was reacted with 9.6 × 10⁻⁴ M FP in 0.05 M Tris-HCl buffer, pH 8, in the dark at room temperature. Activity measurements were performed on aliquots of the reaction mixture, 100-fold diluted at time 0 in 0.05 M Tris-HCl buffer, pH 8 (columns 1 and 2), or in the same buffer containing 5 × 10⁻⁴ M pyridoxal-P (columns 3 and 4). Time 0 represents the activity in the absence of added FP. 1. Specific activity 5 min after dilution; 2, the same as 24 h after dilution; 3, specific activity in the presence of pyridoxal-P 30 min after dilution; 4, the same as 24 h after dilution.

| Dilution time | No PLP added | PLP added |
|---------------|--------------|-----------|
| 5 min         | 1            | 3, 4      |
| 24 h          | 2            |           |
| 30 min        | 3            |           |
| 24 h          | 4            |           |
| 3 h           | 6            |           |
| 12 min        | 22           | 68        |
| 30 min        | 24           | 62        |
| 60 min        | 32           | 65        |
| 90 min        | 34           | 65        |
| 2 h           | 40           | 65        |
| 3 h           | 46           | 69        |
| 6 h           | 68           | 71        |

Activity measurements without addition of pyridoxal-P showed that the spontaneous reactivation reached a maximum of 30% of the activity of untreated reconstituted holoenzyme in 6 h.

The shape of the spectra at 24 h of reaction, after elimination of unbound reagent by gel filtration, suggests the presence of enzyme in aldaminic form.

Evidence for the formation of pyridoxal-P during the reaction was obtained by addition of the amino acid substrate cysteine sulfinic acid (see "Experimental Procedures"). Spec-
tral variations were obtained, indicating the shift from the aldehydic to the amionic form of the coenzyme.

From the known molar extinction coefficients of amionic and aldehydic holoenzyme (27), 28% of coenzyme content was calculated, in good agreement with activity measurements. Furthermore, when this transaminated protein was denatured, the spectral features of the resulting supernatant were typical in shape and pH dependence of pyridoxamine-5'-phosphate.

The spectral features of the denatured protein are shown in Fig. 7B. The shape of the curve and the absorption above 300 nm, when compared with similarly treated cytoplasmic apoenzyme, suggest that no specific labeling has occurred.

**DISCUSSION**

The reagent FP has a great affinity for the coenzyme binding site of both aspartate aminotransferase isoenzymes from pig heart, as shown by the strong inhibition of the binding site of both aspartate aminotransferase isoenzymes pseudo-substrates label in both cases the active site lysyl residue (9, 10).

In this report, it is demonstrated that FP, in the case of the cytoplasmic isoenzyme, covalently reacts with the active site lysine and behaves, therefore, as a typical affinity labeling reagent, while in the case of the mitochondrial isoenzyme, it is unable to specifically label either the lysyl residue or any other group so that the treated enzyme can be completely reactivated under proper experimental conditions.

The lysyl amino group at the active site is bound in the holoenzyme to the aldehydic carbon in 4' position of the coenzyme. The same amino group in order to bind to the reagent must react with a carbon atom of the fluorodinitrophenyl moiety which, in the enzyme, is placed at a distance of a few angstroms from the position normally occupied by the 4' carbon atom of the coenzyme. This binding, therefore, demonstrates a great mobility in lysine 258 of the cytoplasmic isoenzyme, in accordance with the data of Morino and Tanase (10) who showed that the amino group of this lysine can react with the β carbon atom of a pseudo-substrate bound to the holoenzyme. The failure of the mitochondrial isoenzyme to undergo the same reaction might be due to a restricted mobility of its active site lysyl residue or to a substantially different conformation of the reagent at the active site. The reagent, for instance, could be positioned so that it projects its reactive fluorodinitrophenyl moiety toward the exterior of the protein and away from other nucleophiles. In both cases, a definite difference in the active sites of the two isoenzymes must be present.

FP appears to be a very sensitive indicator of such a difference, which is presumed to be rather small. A similar conclusion was reached by Okamoto and Morino (28), who showed that in the reaction of the two holoenzymes with β-bromopropionate, only the mitochondrial isoenzyme is inactivated.

The formation of the reversible inhibitor-apoenzyme complex has another consequence (beside the possible covalent labeling of a protein side chain), namely the cleavage of the inhibitor with the consequent formation at the active site of pyridoxal-P and, hence, reactivation. This unwanted reaction in our case is not fast enough to affect the labeling potential of the reagent, as shown by the results obtained with the cytoplasmic enzyme. On the other hand, the same reaction cannot be held responsible for the lack of labeling in the mitochondrial enzyme, since in 6 h it reaches only 30%.

In the cytoplasmic isoenzyme, a labeling reaction occurs also on the protein surface, leading to the covalent binding of the inhibitor to the sulphydryl group of cysteine 45.2

Since the same reaction occurs also in the holoenzyme, it appears that there is no need for the inhibitor to be bound at the active site in order to react with cysteine 45 so that the labeling does not take place by an affinity mechanism.

Experiments performed with the dephosphorylated analog of FP show that the same cysteine 45 reacts at a comparable rate, while little binding of lysine 258 is noticed in the apoenzyme (12).

It can be concluded that the lysine 258 modification occurs by affinity labeling and that FP is able to reveal slight differences in the active sites of two isoenzymes; the amino acid side chains of histidine and tyrosine, which, at least for the cytoplasmic isoenzyme, are supposed to be at the active site (29), are placed in such a position as to be unaccessible to FP.

The high affinity of the reagent for the coenzyme binding site of transaminases makes it a potentially useful tool also for the study of other pyridoxal-P-dependent enzymes.

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