The Metabolism of Mitochondrial Proteins

I. DISTRIBUTION AND CHARACTERIZATION OF THE ISOZYMES OF ALANINE AMINOTRANSFERASE IN RAT LIVER*

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In 1963, the existence of two isozymes of alanine aminotransferase (L-alanine:2-oxoglutarate aminotransferase, EC 2.6.1.2) was still somewhat in doubt. Hird and Rowsell (2) reported that alanine aminotransferase was present in the particulate fraction as well as in the soluble phase of rat liver homogenates, and they speculated that the activity was of mitochondrial origin. However, in studies of the release of aminotransferases into serum following liver injury, Fleisher and Wakim (3), using electrophoresis, found only a single alanine aminotransferase, although they discerned both isozymes of aspartate aminotransferase. Segal, Beattie, and Hopper (4), in an extensive study of hepatic alanine aminotransferase, detected only a soluble form of the enzyme. Nevertheless, reports of the existence of a mitochondrial enzyme continue to be published. Kafer and Pollak (5) found 20% of the total alanine aminotransferase activity of liver homogenates in the mitochondrial fraction, and Katunuma et al. (6) reported finding 15%. Rowsell, Turner, and Carmie (7) subsequently found only 4 to 5% of the total activity in the particulate fraction.

Because of the disparity of these results, we chose to re-examine the question of the mitochondrial isozyme of alanine aminotransferase. Fractional and gradient centrifugation experiments indicated that the enzyme was present in the mitochondrial as well as in the supernatant fraction, albeit in relatively low concentration. An attempt was therefore made to enrich the isozymes and to characterize them chemically. Purification of alanine aminotransferase from a rat liver homogenate was described by Segal et al. (4), and we encountered little difficulty in repeating their experiments with the enzyme found in the supernatant fraction. More recently, Hopper and Segal (8) recorded attempts to study the mitochondrial enzyme from rat liver with results similar to ours. The enzyme is extremely labile under most conditions, and little enrichment has been achieved. The present report describes the solubilization of mitochondrial alanine aminotransferase by treatment with glycerol or hexadecyltrimethylammonium bromide and its stabilization by glycerol and related compounds. Estimates of the kinetic parameters have been made with the use of such relatively crude, stabilized preparations. It now seems clear, from the extreme difference in stability and from the kinetic data recorded here, that two isozymes of alanine aminotransferase are indeed present in rat liver.

Modification of the activity of both isozymes in the livers of rats treated with hormones or fed diets of differing protein composition is described in the accompanying paper (9).

EXPERIMENTAL PROCEDURE

Adult female rats, obtained from Badger Research Laboratories or of the SD/Anl-SPF strain, were maintained on Lab-Blox and were killed by decapitation. The livers were quickly removed and chilled in ice for 10 min.

When it became apparent that the mitochondrial isozyme of alanine aminotransferase was quite unstable, it was necessary to modify the usual methods of preparation of cellular fractions and of exposure of mitochondrial enzymes for assay purposes. The following procedure, in our hands, has given the highest and most consistent estimates of alanine aminotransferase activity in rat liver mitochondria. A 10% homogenate in 0.25 M sucrose-0.01 M K2HPO4 was prepared with the use of a Potter-Elvehjem homogenizer with a Teflon pestle; a centrifugation procedure adapted from that of Schneider and Hogeboom (10) was used for the enrichment of the particulate fractions. The various pellets were resuspended twice in buffered sucrose, stirred vigorously by repeated aspirations into a 20-ml syringe, and centrifuged. The final pellets were suspended in a medium containing 0.05 M potassium phosphate buffer (pH 7.4), 0.025 M L-alanine, and 0.002 M L-cysteine. Alanine apparently stabilized the mitochondrial enzyme and was the only one of its four substrates active in this regard. Cysteine hastened the swelling of mitochondria (11) and made it possible to assay for alanine aminotransferase activity 30 to 60 min after preparation. Otherwise, maximal, but uncertain, activity was obtained only after standing at 0° for 1 to 3 hours.†

Because mitochondrial alanine aminotransferase activity was recorded here, that two isozymes of alanine aminotransferase are indeed present in rat liver.

† The employment of techniques frequently used to render mitochondrial enzymes more accessible to their substrates, such as freeze-thawing, sonic disintegration, or incubation with detergents, failed to enhance the apparent particulate alanine aminotransferase activity. Although these treatments undoubtedly exposed more enzyme, they apparently also accelerated its decomposition. It was found that glycerol and hexadecyltrimethylammonium bromide would effect the solubilization of mitochondrial alanine aminotransferase; however, suspension of intact mitochondria in 90% glycerol or in 0.1% hexadecyltrimethylammonium bromide before assay failed to enhance the apparent specific activity because both compounds also inhibited the enzyme: 11 to 23% and 15 to 37%, respectively.

3334
The data (input) obtained from several kinds of assays and analyses automatically control the computations made, and programs written with the kind assistance of Mr. Merlin Dipert. A program for calculation of specific activities, in which Fortran programs were performed by a digital computer with the kind assistance of Mr. Merlin Dipert. A program for calculation of specific activities, in which the data (input) obtained from several kinds of assays and analyses automatically control the computations made, and programs for calculation of apparent and absolute Michaelis constants are available from the authors.

The assay of alanine aminotransferase activity found in the supranatant fraction (hereafter known as the soluble enzyme) could be linked directly to the oxidation of NADH (13), and the procedure described in Fig. 1 was used during the purification of the soluble enzyme. However, the low activity of the mitochondrial isozyme relative to the activity of other enzymes catalyzing the oxidation of NADH precluded routine use of the coupled assay. Hence, an interrupted assay as described in Fig. 2 was used for estimation of alanine aminotransferase activity in the mitochondrial pellet as well as in the other fractions in most of the experiments. Essentially the same values for the activity of soluble alanine aminotransferase could be obtained by either of the two methods. Although less certain because of the large corrections required in the coupled assay, solubilized preparations of the mitochondrial enzyme possessed similar activities when assayed by either method.

The reverse reaction was also measured in an interrupted assay with the use of pyruvate (26 μmoles) and L-glutamate (100 μmoles). The α-ketoglutarate formed was estimated from the oxidation of NADH by glutamate dehydrogenase.

Concentrations of the various substrates which would give initial zero order reaction rates were determined by varying independently the amount of each substrate until optimum conditions obtained. Figs. 1 and 2 show the amount of pyruvate formed plotted against time for four levels of enzyme. Similar plots are obtained when the pyruvate formed is plotted against the amount of enzyme added for three time intervals. Protein concentration was measured by the method of Lowry et al. (14), and specific activity was expressed as micromoles of product formed per min per mg of protein.

RESULTS

Distribution—Enzyme assays for both succinate dehydrogenase and alanine aminotransferase activity were carried out on the 13 fractions obtained by gradient centrifugation. Succinate dehydrogenase, an enzyme found only in the mitochondria, was used to locate the highest concentration of mitochondria. Fig. 3A shows the distribution of alanine aminotransferase and succinate dehydrogenase activity in a whole homogenate. Most of the alanine aminotransferase activity was found in the nonparticulate phase, but a small peak of activity coinciding with the fractions richest in succinate dehydrogenase was also present. A mitochondrial fraction first prepared by differential centrifugation was resuspended in sucrose and centrifuged in a gradient. Fig. 3B shows the distribution of the two enzymes in the fractions obtained. Although there still appears to be some contamination with the soluble enzyme, the peaks of activity coincide exactly, suggesting that alanine aminotransferase activity is definitely associated with this particle. However, because the mitochondria were found to contain such a small proportion of the total enzyme activity, it remained uncertain from these experiments whether this activity was due to the presence of a mitochondrial isozyme of alanine aminotransferase or to some type of association of the soluble enzyme with this cellular component.

Enzyme assays were also carried out in Tris buffer on the five fractions obtained by conventional differential centrifugation of sucrose homogenates. The specific and total activity of the nuclear, microsomal, and "poorly sedimentable" (15) layers was quite low and was probably the result of contamination (Table 1). The specific activity of the mitochondrial alanine aminotransferase was higher and accounted for about 8% of the total activity of the mitochondrial enzyme. The specific activities obtained are given in Table 1.
FIG. 2. Proportionality of micromoles of pyruvate formed in an interrupted assay to incubation time at four levels of a mitochondrial suspension. Conditions: 400 μmoles of Tris buffer (pH 8.0) (or 400 μmoles of potassium phosphate buffer, pH 7.6), 200 μmoles of L-alanine, and 40 μmoles of α-ketoglutarate were equilibrated at 30° for 5 min, and an aliquot of the fraction to be assayed was added (final volume, 4 ml). At intervals, aliquots were pipetted into 20% trichloroacetic acid and centrifuged. An aliquot of the supernatant solution and 300 μmoles of potassium phosphate were adjusted to pH 7.2 with 0.1 M alkali; 0.4 μ mole of NADH was added, and the adsorption at 340 μm was read. The amount of pyruvate formed was measured from the oxidation of NADH catalyzed by the subsequent addition of lactate dehydrogenase.

Alanine aminotransferase activity was higher in all fractions prepared from the liver of a rat given prednisolone: that of the mitochondria was about 34 times that of the control rat, while the specific activity of the soluble enzyme was 24 times as high (Table I). The increase in the activity of mitochondrial alanine aminotransferase has been obtained consistently, and this increase is in contrast to the results reported by Hopper and Segal (8) and by Takeda et al. (16), who found no change in the specific activity of hepatic mitochondrial enzyme from corticosteroid-treated rats. The effects of hormone treatment are considered in detail in the accompanying paper (9).

Preparation of Soluble Enzyme—Livers from rats treated with injections of prednisolone for several days preceding death were used as a rich source of soluble alanine aminotransferase. After preparation of the mitochondria, the supernatant fraction was obtained by centrifugation at 63,000 x g for 1 hour. Enrichment by Purification Procedure II of Segal et al. (4) was not carried beyond elution from DEAE-cellulose with 0.1 M NaCl because adsorption and elution from calcium phosphate gel gave erratic results. When assayed at 30°, the final preparation had a specific activity of 44 μmoles per min per mg of protein and was used for both characterization and kinetic studies without further treatment.

Preparation and Stability of Mitochondrial Enzyme—Mitochondria were prepared as described above from the livers of normal rats and from rats previously treated with injections of prednisolone. Solubilization of alanine aminotransferase in good yield was difficult. Freeze-thawing at either -195° or -10°, sonic treatment in a Raytheon 10-Kc sonic oscillator for 1 to 5 min, incubation with Triton X-100 (0.1%), or suspension in water or dilute buffer produced either poor or inconsistent amounts of extracted enzyme, or both. Release of the enzyme was accomplished, however, by the following procedure. The mitochondria were suspended in a 1:1 mixture of glycerol and 0.05 M phosphate buffer (pH 7.4), homogenized briefly or stirred for 10 to 15 min at 0°, and centrifuged at 25,000 x g for 30 min. The supernatant layer was carefully removed and discarded; the pellet was resuspended with rapid mixing in the phosphate-amino acid buffer. After centrifugation, the supernatant layer contained 40 to 65% of the total alanine aminotransferase activity assayed initially, and the specific activity ranged from 38 to 202% (average, 136%) of the initial specific activity. Varying the concentration of glycerol in the suspending medium in either direction gave poorer results. Suspension in 100% glycerol followed by dilution to 5% also gave a poor yield of enzyme.

This procedure was compared with other methods for the solubilization of a number of mitochondrial enzymes in order to determine its general applicability. Mitochondrial malate dehydrogenase, aspartate aminotransferase, glutamate dehydrogenase, and 3-hydroxybutyrate dehydrogenase were assayed in the supernatant fractions obtained by glycerol treatment, sonic disintegration for 1 min, freezing to -195° and thawing to 0° one time, or incubation with Triton X-100 (0.1%) at room temperature. The specific activities of the various enzymes prepared by treatment with glycerol were equal to or higher than those obtained by the other methods. Slightly lower yields of enzyme activity resulted because of losses incurred during the initial wash with the 60% glycerol solution.
Similar solubilization of alanine aminotransferase could be accomplished by replacing glycerol with dimethyl sulfoxide. A 30% solution was optimum, but the yield was usually about 40% of the total activity with some enhancement of the specific activity. Later it was found that suspension of the mitochondria in a 0.1% solution of hexadecyltrimethylammonium bromide released 60 to 75% of the enzyme activity with little or no increase in apparent specific activity. Treatment with glycerol had the added advantage that alanine aminotransferase activity is stabilized by glycerol.

Alanine aminotransferase found in the supernatant fraction is stable in either crude or purified form for long periods (more than 2 months) when stored at 0°C. On the other hand, the mitochondrial isozyme has a half-life of about 6 hours. None of the usual procedures for stabilization of enzymes retarded its inactivation; indeed, most manipulations hastened it. The addition of alanine (0.1 to 0.2 M) and of its next homologue, α-amino-butyrate (0.1 M), doubled the half-life, and storage of solubilized alanine aminotransferase in glycerol (70%), erythritol (saturated, approximately 40%), or dimethyl sulfoxide (50%) preserved most of the enzyme activity for 4 days. Even after 10 days in glycerol, 68% of the activity remained. The mechanism of action of these compounds is unknown.

It appears unlikely that inactivation is a result of dissociation of the coenzyme. Neither incubation of the enzyme with 0.1 or 10 μmoles of pyridoxal phosphate for 5 to 15 min before an assay nor the addition of pyridoxal phosphate to an assay mixture restored activity to old preparations.

Attempts to Purify Mitochondrial Enzyme—All efforts to enrich the enzyme by conventional means so far have proved futile because of its marked instability. None of the initial steps effective for the isolation of the soluble enzyme accomplished any enrichment of mitochondrial alanine aminotransferase. It was possible to precipitate an active fraction between 40 and 65% saturation with ammonium sulfate, but little enhancement in specific activity was achieved, and only about 50% of the enzyme was recovered. Only a trace of enzyme activity remained after dialysis, starch block electrophoresis, chromatography on Sephadex G-75, or precipitation with cold ethanol or acetone.

Stabilization in glycerol or dimethyl sulfoxide led to a small decrease. The viscosity of the solutions precluded the use of chromatographic purification procedures, and no protein was precipitated when a glycerol solution of the enzyme was saturated with ammonium sulfate. MgSO₄ and LiCl were also tried as activating agents, but they were of no help. Only a trace of enzyme activity remained after dialysis, starch block electrophoresis, chromatography on Sephadex G-75, or precipitation with cold ethanol or acetone.

### Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Normal</th>
<th>Prednisolone-treated</th>
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<tbody>
<tr>
<td></td>
<td>Specific activity</td>
<td>Total activity</td>
</tr>
<tr>
<td>Nuclei</td>
<td>μmoles/min/ mg protein</td>
<td>μmoles/min/ g liver</td>
</tr>
<tr>
<td>Poorly sedimentable fraction (15)</td>
<td>0.022</td>
<td>0.16</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.001</td>
<td>0.03</td>
</tr>
<tr>
<td>Supernatant fraction</td>
<td>0.288</td>
<td>2.31</td>
</tr>
<tr>
<td></td>
<td>0.298</td>
<td>2.38</td>
</tr>
<tr>
<td>Total</td>
<td>0.025</td>
<td>0.04</td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.131</td>
<td>28.30</td>
</tr>
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aminotransferase was most active between pH 7.4 and 8.2 (Fig. 4A) while the soluble enzyme had an optimum range of pH 7.3 to 7.8 (Fig. 4B). Similar results were obtained by Segal et al. (4) for the soluble enzyme; however, Katunuma et al. (6) reported the optimum pH for the mitochondrial isozyme as 8.5 and that for the soluble enzyme as pH 9.7.

The enzymes can be distinguished by the difference in their activities when assayed in various buffers (Table II). In an assay with alanine and α-ketoglutarate as substrates, the mito-

TABLE III

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>Ratio of forward to reverse reaction rates measured in</th>
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<tbody>
<tr>
<td></td>
<td>Phosphate</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>3.0</td>
</tr>
<tr>
<td>Soluble</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Fig. 5. Kinetics measured by an uncoupled assay with α-ketoglutarate (aKG) as the variable substrate. The medium contained 300 μmoles of sodium pyrophosphate, pH 7.8, and L-alanine at the levels shown on the curves. v is expressed as micromoles of pyruvate formed per min; v/(α-ketoglutarate) was calculated from the millimolar concentration of α-ketoglutarate. The curve in the inset shows the extrapolation of the apparent Michaelis constants (Kₐᵥ) to infinite L-alanine concentration (ALA). A, alanine aminotransferase prepared from liver mitochondria of a normal rat; B, alanine aminotransferase prepared from liver mitochondria of a rat treated with injections of 2 mg of prednisolone each day for 3 days before death.
Mitochondrial enzyme was equally active in phosphate or Tris buffer but only about one-half as active in pyrophosphate buffer. The soluble enzyme was equally active in phosphate or pyrophosphate but more active in Tris. Similar results were obtained with pyruvate and glutamate as substrates except that the activity of both forms was lower in pyrophosphate than in phosphate buffer.

Rates of Forward and Reverse Reactions—There was no pronounced effect of the kind of buffer used on the ratio of the initial rate of the forward reaction to that of the reverse reaction (forward reaction: alanine + \( \alpha \)-ketoglutarate \( \rightarrow \) pyruvate + glutamate). The forward reaction was more rapid in all cases and ranged, for the mitochondrial enzyme, from 3.0 to 4.9 times as fast and, for the soluble enzyme, from 1.2 to 2.1 times the rate of the reverse reaction (Table III). However, when the approach to equilibrium was measured from both directions by estimation of pyruvate formation or disappearance, a value of 0.6 was obtained for both isozymes. Similar results were reported by Segal et al. (4).

Michaelis Constants—Estimates were made of the apparent Michaelis constants for alanine and for \( \alpha \)-ketoglutarate at three levels of the cosubstrate for the soluble enzyme and for mitochondrial alanine aminotransferase prepared from normal rats and from rats previously treated with injections of prednisolone. Although a coupled assay was used to study the soluble enzyme while an interrupted assay was necessary with mitochondrial preparations, the results appear comparable since the two procedures give similar results in routine estimates of isozyme activity. Figs. 5 and 6 give examples of the data obtained with mitochondrial preparations. A secondary plot of the reciprocals of the apparent constants against the reciprocals of the cosubstrate concentrations permits estimation of the absolute Michaelis constant at infinite cosubstrate concentration. The values obtained are summarized in Table IV. The constants calculated for the soluble enzyme agree quite well with those presented by Hopper and Segal (17) for alanine aminotransferase prepared from a rat liver homogenate. The absolute Michaelis constant for \( \alpha \)-ketoglutarate was similar for the isozymes, but the constant for alanine for the mitochondrial enzyme was much lower than that obtained for the soluble form. The values for both substrates found for the mitochondrial isozyme prepared from rats treated with prednisolone were like those calculated for the enzyme prepared from normal animals (Table IV).

**FIG. 6.** Kinetics measured by an uncoupled assay with L-alanine (ALA) as the variable substrate. The medium contained 300 \( \mu \)moles of sodium pyrophosphate, pH 7.8, and \( \alpha \)-ketoglutarate at the levels shown on the curves. \( v \) is expressed as micromoles of pyruvate formed per min; \( v/\text{(ALA)} \) was calculated from the millimolar concentration of L-alanine. The curve in the inset shows the extrapolation of the apparent Michaelis constants (\( K'_{\text{m}} \)) to infinite \( \alpha \)-ketoglutarate concentration (\( eKG \)). A, alanine aminotransferase prepared from liver mitochondria of a normal rat; B, alanine aminotransferase prepared from liver mitochondria of a rat treated with injections of 2 mg of prednisolone each day for 3 days before being killed.

**DISCUSSION**

Because of the relatively small amounts of alanine aminotransferase found in the mitochondria, the failure of some workers to find any activity associated with these particles (perhaps partly as a result of lability), and the initial findings in our laboratory that the change in mitochondrial activity parallels that of the activity of the supernatant fraction after hormone injection, the existence of a true isozyme of alanine aminotransferase in the mitochondria was in doubt. However, its presence seems assured in light of several differences between the two forms which have now been found. The marked instability of the mitochondrial enzyme first noted by Hopper and Segal (8) and also reported here, the success of Katunuma et al. (6) and Takeda et al. (16) in separating the isozymes chromatographically, the differences in the inducibility of the two enzymes (9), the differences in activity in various buffers, and the differences in the

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**TABLE IV**

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>Absolute Michaelis constant for</th>
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<tr>
<td></td>
<td>Alanine</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td></td>
</tr>
<tr>
<td>Normal rat</td>
<td>11.0 ± 0.0</td>
</tr>
<tr>
<td>Hormone-treated</td>
<td>9.2 ± 0.7</td>
</tr>
<tr>
<td>Soluble</td>
<td>42.0 ± 5.6</td>
</tr>
</tbody>
</table>

* Values given by Hopper and Segal (17) for alanine aminotransferase prepared from a rat liver homogenate.
Michaelis constants for alanine support the idea that two proteins with alanine aminotransferase activity are present in rat liver.

Intensive studies (17-19) of the aminotransferase reactions have led to the postulation of a mechanism that involves the occupation of a single catalytic site on the enzyme by each of the substrates, one at a time and in sequence. The evidence that the reaction is essentially monomolecular rests partly on the observation that the ratio of the apparent Michaelis constant to the maximum velocity is independent of the cosubstrate concentration and is constant. This ratio is represented by the intercept on the ordinate in Figs. 5 and 6. Since the intercepts are quite similar for different cosubstrate concentrations in any given experiment, it appears likely that the mitochondrial isozyme of alanine aminotransferase also operates by this mechanism.

Two factors suggest that mitochondrial alanine aminotransferase favors pyruvate (or glutamate) formation in vivo. The absolute Michaelis constant for alanine is about one-fourth that for the soluble enzyme; i.e. the enzyme operates with a much lower concentration of alanine than does the soluble isozyme. A similar difference in the Michaelis constants for aspartate has been shown for the isozymes of aspartate aminotransferase (20, 21). Second, the reaction catalyzed by the mitochondrial enzyme goes more rapidly toward pyruvate than the one catalyzed by the soluble isozyme, although the equilibrium of the reaction for both enzymes favors alanine (and α-ketoglutarate) formation. Reactions in vivo, however, rarely approach equilibrium.

Increases in the activity of other enzymes following hormone treatment have been shown to be the result of an increase in the amount of enzyme protein (22-25). The data presented here are consistent with the hypothesis that the increase in mitochondrial alanine aminotransferase activity following the injection of prednisolone is the result of an increase in the amount of (mitochondrial) enzyme protein in the particle. The Michaelis constants for alanine and α-ketoglutarate for enzyme prepared from hormone-treated animals are unchanged. Secondly, mitochondrial alanine aminotransferase prepared from treated animals is as unstable as that isolated from normal rats; i.e. there has been no activation or modification of the enzyme nor has there been any incorporation of (or preparative contamination with) the soluble enzyme. Final proof must await purification of the enzyme so that appropriate immunological or isotope experiments can be performed.

Assay of other rat tissues for mitochondrial alanine aminotransferase showed it to be present but in very low concentration. Therefore, none of these tissues offered an alternative to the liver as a useful source of the rat enzyme. Katumuma et al. (6) prepared the mitochondrial isozyme from pig heart, and we have recently found that alanine aminotransferase prepared from rabbit liver mitochondria is reasonably stable. Further studies of this enzyme will probably have to involve its preparation from one of these or other sources.

The existence of a mitochondrial isozyme of alanine aminotransferase has been demonstrated. It represents about 8% of the total alanine aminotransferase activity in rat liver, and its activity can be increased several fold by the administration of prednisolone. The mitochondrial enzyme was solubilized by treatment with glycerol or hexadecytrimethylammonium bromide and stabilized by the addition of glycerol or dimethylsulfoxide. It could be distinguished from the major alanine aminotransferase component found in the soluble phase by its marked lability, by the differences in its activity in various buffers, and by a much smaller absolute Michaelis constant for alanine.

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