ON THE MECHANISM OF ACTION OF GLUTAMIC-ASPARTIC TRANSAMINASE: INTERMEDIATE STEPS IN THE REACTION*

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SUMMARY

Substrate amounts of highly purified GAT react with glutamate, aspartate and, at a much slower rate, with alanine, but not with glycine, arginine, serine and isoleucine, to give the corresponding ketoacids. The absorption spectrum of the enzyme changes during the reaction and the coenzyme passes from the aldehydic (enzyme–PLP) to the aminic (enzymic–PMP) form. Enzyme–PMP was isolated from the reaction mixture and its spectrum was studied at different pH values. The coenzyme/protein ratio and the specific activity of enzyme–PMP were the same as those of enzyme–PLP. The aminic form of the enzyme reacts with ketoglutarate, oxalacetate and, at a slower rate, with pyruvate, to give the corresponding amino acids and the aldehydic form of the enzyme.

INTRODUCTION

It is generally assumed (1–4) that the mechanism of enzymic transamination may be represented by the following equations:

$$\text{Enzyme–PLP} + \text{amino acid I} \leftrightharpoons \text{enzyme–PMP} + \text{keto acid I} \quad (1)$$

$$\text{Enzyme–PMP} + \text{keto acid II} \leftrightharpoons \text{enzyme–PLP} + \text{amino acid II} \quad (2)$$

This hypothesis was first substantiated by SNELL *et al.*[4] in their extensive work on model reactions and it has been recently confirmed by studies on purified enzymes. MEISTER *et al.*[5] showed that both PLP and PMP can activate the GAT system. However, most of the attempts to demonstrate the conversion of enzyme–PLP to enzyme–PMP were not successful[3] until JENKINS *et al.*[6,7] not only confirmed the presence of PLP in GAT from pig heart, but, in a preliminary note[8], reported also that, after the addition of glutamate to the enzyme, the prosthetic group was transformed into PMP, and that the reaction was reversed by an excess of α-ketoglutarate.

Abbreviations: GAT, glutamic–aspartic transaminase; MDH, malic dehydrogenase; LDH, lactic dehydrogenase; enzyme–PLP, aldehydic form of transaminase; enzyme–PMP, aminic form of transaminase; PLP, pyridoxal-5-phosphate; PMP, pyridoxamine-5-phosphate; DPNH, reduced diphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide.

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In the work here described the reaction of highly purified GAT with several amino acids was investigated. Moreover, enzyme–PMP was isolated, so that it has been possible to study also the reaction of enzyme–PMP with oxalacetate, ketoglutarate and other keto acids.

EXPERIMENTAL

Materials

γ-amino acids were from Merck and Co., Darmstadt (Germany); oxalacetic acid from California Foundation for Biochemical Research, Los Angeles (U.S.A.); α-ketoglutaric acid from Sigma Chemical Co., St. Louis (U.S.A.); PLP from Hoffmann-La Roche, Basel (Switzerland); PMP was prepared according to PETERSON AND SOBER[9].

GAT was prepared from pig heart according to LIS[10] with minor modifications[11]. MDH was prepared together with GAT[11]. LDH and DPNH were from Böhringer and Sohn (Germany).

Methods

Activity measurements: GAT activity was determined as previously described[10] with the minor modification that the 10-min incubation of the aminoacid and enzyme before the addition of the keto acid was omitted.

Proteins were determined by the biuret method[12] and by their absorbancy at 280 mμ in 0.1 *M* phosphate buffer (pH 7), after having determined the specific extinction coefficient at that wavelength by reference to the dry weight of the protein, after heating at 110° in the air until constant weight is reached.

Determination of coenzymes: Coenzymes were removed from the protein by solving the enzyme in 0.1 *N* NaOH or KOH. For the qualitative identification of both forms of the coenzyme, the KOH solution of the enzyme was neutralized with $HClO_4$; the precipitated $KClO_4$ and denatured protein were removed by centrifugation and the supernatant was lyophilized, dissolved in a minimum of water and analysed by paper chromatography[13] and electrophoresis[14].

PLP was quantitatively determined on the basis of its absorption at 388 mμ in the 0.1 *N* NaOH solution[6,15]. For the quantitative determination of PMP the 0.1 *N* NaOH solution of the enzyme was brought to pH 4.6 with acetic acid. This caused the precipitation of denatured protein, which was removed by centrifugation; the clear supernatant fluid was brought to pH 3.2 with 1 *M* citric acid and placed on the top of a Dowex 50 column, 200–300 mesh, (4 × 0.7 cm) in the Na^+-form, equilibrated with citrate buffer (pH 3.2) 0.1 *M* respecting Na^+. The column was washed with 15 ml of the same citrate buffer. PMP was eluted from the column in 0.1 *M* sodium acetate. PMP was usually completely recovered in the first 5 ml of the elution fluid; this was successively brought to pH 7 and used for the fluorometric determination of PMP in a Farrand spectrofluorometer, using an exciting light at 330 mμ and analysing at 390 mμ as previously described[16]. Controls of the chromatographic behaviour and recovery were made with pure PMP, both alone and added to the enzyme extract.

Preparation of enzyme–PLP: A solution of the enzyme obtained according to LIS[10,11] was added, with ketoglutarate, to a final concentration of 0.01 *M*, brought to pH 5.4 with 1 *N* acetic acid and precipitated in the cold with 1 volume of acetone. After

centrifugation the supernatant was discarded and the enzyme was dissolved in 0.01 *M* acetate buffer (pH 5.4) in a volume corresponding to one half of the original solution; the enzyme was then again precipitated with acetone. This treatment was repeated twice in order to remove the remaining traces of the keto acid. Acetone was eliminated from the final precipitate by gentle aeration in the cold. The protein was then dissolved in the desired buffer at the desired concentration and the solution obtained was centrifuged again in order to remove traces of insoluble material.

Reaction between enzyme–PLP and amino acids

Reaction mixture: To a solution of enzyme–PLP in 0.08 *M* pyrophosphate buffer (pH 8.4), a 1 *M* amino acid solution (natrium salt) in the same buffer was added to a final concentration of 0.3 *M*. The final concentration of the enzyme was 0.5%. Sparingly soluble amino acids (*i.e.* serine, isoleucine, arginine) were added as saturated solutions to a final concentration of 0.02 *M*. The absorption spectra of the solution were determined before and after the addition of the amino acid.

In experiments for the quantitative determination of oxalacetate and pyruvate, the reaction was carried out in 0.02 *M* phosphate buffer (pH 7.4).

Determination of oxalacetate: For the determination of oxalacetate, the MDH system proposed by KARMEN[15] was used. To the enzyme–PLP–aspartate reaction mixture was added 1/60 volume of the MDH solution (0.1 mg protein/ml) and 1/60 volume of the DPNH solution (5 μmoles/ml). A blank was carried out containing all the components excepting GAT. The increase of the absorbancy at 340 mμ upon addition of DPNH was measured in the test and blank solutions by means of a Beckman D.U. Spectrophotometer. The amount of oxalacetate was calculated from the difference in the increase of absorbancy in the test and blank. A preliminary control had shown that no oxalacetate was formed when aspartate was omitted from the reaction mixture.

Determination of pyruvate: Pyruvate was determined in the GAT–alanine reaction mixture by the method described by BAUER[18]. The experimental procedure was the same as the one described above for oxalacetate, excepting that MDH was replaced by LDH.

Determination of other ketoacids: The enzyme was precipitated with acetone from the enzyme–PLP–glutamate (or other amino acid) reaction mixture and the keto acid was determined in the supernatant by the method of CAVALLINI AND MONDOVI[19] after removal of acetone by distillation under vacuum.

Preparation of enzyme–PMP: The enzyme was recovered from the GAT–glutamate (or aspartate) reaction mixture by precipitation with acetone at pH 5.4. The precipitate was dissolved in a volume of 0.01 *M* acetate buffer (pH 5.4) equal to that of the original solution and precipitated with 1 volume of acetone. The treatment was repeated twice in order to remove the remaining traces of the amino acid. The final precipitate was gently aerated in the cold so as to remove acetone, and then solved in the desired buffer.

Reaction between enzyme–PMP and ketoacids

0.05 *M* ketoacid (sodium salt, in 0.08 *M* pyrophosphate buffer (pH 8.4) was added to the enzyme–PMP solution in a final concentration of 0.01 *M* ketoacid and 0.5 % protein.

The absorption spectrum was recorded before and after the addition. After 10 min at room temperature, the enzyme was precipitated with acetone; the supernatant solution was concentrated under vacuum and the amino acids present in it were determined according to ASPEN *et al.*[20].

RESULTS AND DISCUSSION

Coenzyme content, specific activity and absorption spectra

The coenzyme content and specific activity of the GAT preparation, obtained by the method described by LIS[10,11], of enzyme–PLP and of enzyme–PMP are reported in Table I. As this table shows, some PMP (about 8 % of the total coenzyme) is present in the GAT preparation obtained from the electrophoresis column[10,11]. Because no ketoacid or aminoacid was added during the preparation and, because, under the experimental conditions followed, the two forms are about equally stable, it can be assumed that this is the ratio in which the two forms of the enzyme occur in pig heart.

Only a little PMP remains in the enzyme preparation after treatment with one of its keto acid substrates, while no PLP can be detected in enzyme–PMP. When, however, these data are considered, it should be borne in mind that the fluorometric method employed for the determination of PMP is far more sensitive than the spectrophotometric method employed for PLP, so that, if enzyme–PMP contained traces of PLP, they would escape detection. The coenzyme/protein ratio found for our preparation (17.4 μmole/g protein) is in good agreement with data calculated by JENKINS *et al.*[6] (13.4 to 14.7 μmole/g protein), on the assumption, made by those authors, that their preparation is 81 % pure.

The specific activity of the three enzyme preparations is essentially the same in both directions.

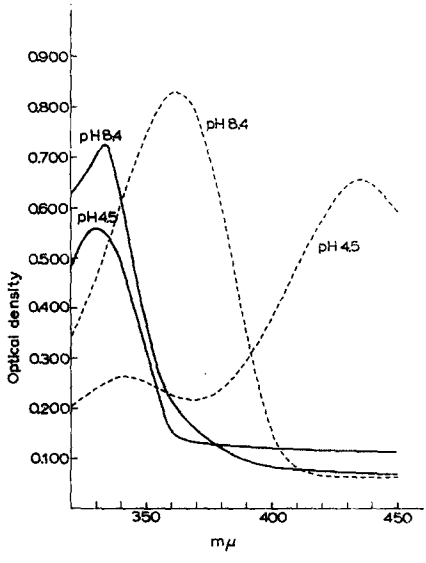

Fig. 1. Absorption spectra of enzyme–PLP (– – – –) and of enzyme–PMP (————) in 0.1 *M* pyrophosphate buffer (pH 8.4) and in 0.1 *M* acetate buffer (pH 4.5). All solutions containe 6 mg of protein /ml.

The absorption spectra of enzyme–PMP and of enzyme–PLP at different pH values are shown in Fig. 1. The pH effect on the spectrum, which is marked on enzyme–PLP[7], is, on enzyme–PMP, very modest.

Reaction between enzyme–PLP and aminoacids

When glutamate or aspartate is added (to a final concentration of 0.3 *M*) the absorption peak of enzyme–PLP at pH 8.4 in the upper u.v. region shifts from 360 to 335 mμ. A similar shift was observed by JENKINS AND SIZER[8] upon addition of glutamate (to a final concentration of 0.5 *M*) to their GAT preparation. Such changes do not occur when glutamate or aspartate are replaced by amino acids on which the enzyme is not active, *i.e.* glycine, isoleucine, serine and arginine. Table II records some quantitative aspects of the reaction between enzyme–PLP and several amino acids.

It is evident from Table II that, when glutamate or aspartate is added to enzyme–PLP to a final concentration of 0.3 *M*, ketoglutarate or, alternatively, oxalacetate appears in the reaction mixture in amounts approximately equivalent to the number of coenzyme molecules bound to the enzyme. The determination of the coenzyme after the addition of either of these amino acids showed that PLP had completely disappeared and that an equivalent amount of PMP had been produced.

TABLE I

SPECIFIC ACTIVITY AND COENZYME CONTENT OF A TYPICAL PREPARATION OF GAT PREPARED ACCORDING TO LIS[10,11], OF ENZYME–PLP AND OF ENZYME–PMP

	PLP (μmoles/g protein)	*PMP (μmoles/g protein)*	*g protein/mole total coenzyme (PLP + PMP)*	*Specific activity (units/mg protein)*	
				Aspartate + ketoglutarate	*Glutamate + oxalacetate*
Enzyme preparation according to LIS[10,11]	15.6	1.4	58,800	5,600	12,000
enzyme–PLP	17.2	0.28	57,200	5,600	11,900
enzyme–PMP	none	17.4	57,400	5,700	12,000

TABLE II

TRANSAMINATION BETWEEN ENZYME–PLP AND AMINO ACIDS

All data are referred to 1 ml of reaction mixture containing 5 mg of enzyme–protein.

μmoles enzyme bound PLP before addition of amino acid	*μmoles amino acid added*	*after addition of the amino acid*		
		μmoles keto acid formed	*μmoles enzyme–PLP*	*μmoles enzyme–PMP*
0.085	300 (glutamate)	0.082 (ketoglutarate)	0.000	0.087
0.087	300 (aspartate)	0.084 (oxalacetate)	0.000	0.084
0.084	20 (aspartate)	not determined	0.046	0.043
0.086	20 (aspartate)*	0.089	0.000	0.088
0.087	300 (alanine)	0.083 (pyruvate)	0.000	0.081
0.086	300 (serine)	none	0.084	
0.086	20 (isoleucine)	none	0.081	
0.085	20 (arginine)	none	0.085	

* in the presence of MDH and DPNH at pH 7.4.

With lower concentrations of the amino acid (*e.g.* 0.02 *M*) both PLP and PMP were found in the alkaline extract of the enzyme. It is of interest to observe that, even with this concentration of glutamate or aspartate, the change of the absorption spectrum at pH 8.4 was complete (*i.e.* identical with the one obtained with 0.3 *M* amino acid). These findings might be interpreted by assuming that the equilibrium of the reaction is pH-dependent: when, that is to say, the pH of the reaction mixture is adjusted to 5.4 in order to precipitate the enzyme with acetone, a part of the keto acid formed at pH 8.4 might react with enzyme–PMP to reconstitute enzyme–PLP and the amino acid.

This hypothesis is in agreement with the observation made by JENKINS[7] that, when the pH is lowered, enzyme–PLP is transformed into the non-active form, which would be subtracted from the equilibrium. Further evidence in support of this interpretation is provided by our finding that, when the reaction of enzyme–PLP with aspartate is carried out at pH 7.4 in the presence of the MDH system (which converts oxalacetate into malate as soon as it is formed and thus subtracts it from the equilibrium) no PLP is found, even when low concentrations of aspartate (0.02 *M*) are used, in the alkaline extract of the enzyme recovered from the reaction mixture by precipitation with acetone at pH 5.4.

It has been mentioned above that some amino acids, on which GAT is not active (*i.e.* serine, glycine, isoleucine and arginine), were unable to modify the spectrum of enzyme–PLP. Their incapacity to react with GAT was confirmed by the finding that, when they were added to the enzyme solution, enzyme-bound PLP was not transformed into PMP and that no keto acid appeared in the reaction mixture (see Table II).

In this connection we have thought it interesting to investigate the behaviour of alanine, which, according to CAMMARATA AND COHEN[21], undergoes transamination with ketoglutarate in the presence of a GAT preparation from pig heart; alanine, however, transaminates at a much lower rate than aspartate. The attribution of this effect to the presence of contaminating traces of glutamic–alanine transaminase could not be excluded. In the present research it was found that, when alanine was added to enzyme–PLP, spectral changes occurred, which were identical with those observed with glutamate and aspartate. Pyruvate, moreover, appeared in the reaction mixture and the enzyme-bound vitamin B_6-derivative turned from PLP into PMP (see Table II).

The reaction of enzyme-PLP with alanine apparently takes place more slowly than the one with aspartate or glutamate, because, when low concentrations of the amino acid (0.02 *M*) are used, the changes in the absorption spectrum, which are instantaneous with glutamate or aspartate, take place so slowly that they can be followed in the spectrophotometer (Table IV).

Reaction between enzyme–PMP and keto acids

When ketoglutarate or oxalacetate was added, the absorption spectrum of enzyme–PMP at pH 8.4 shifted back from a spectrum with a peak at 335 mμ to one with a peak at 360 mμ.

Some quantitative aspects of this reaction are reported in Table III. As this table shows, it was possible to recover glutamate or, alternatively, aspartate from the reaction mixture in amounts equivalent, within the limits of experimental error,

TABLE III

TRANSAMINATION BETWEEN ENZYME–PMP AND KETO ACIDS

All data are referred to 1 ml of the final reaction mixture containing 5 mg enzyme protein.

μmoles enzyme bound PMP before the addition of keto acid	*μmoles keto acid added*	*after addition of the keto acid*		
		*μmoles amino acid formed**	*μmoles enzyme–PMP*	*μmoles enzyme–PLP*
0.088	10 (ketoglutarate)	0.091 (glutamate)	0.012	0.081
0.083	10 (ketoglutarate)	0.079 (glutamate)		0.087
0.084	10 (oxalacetate)	0.095 (aspartate)	0.011	0.085
0.082	10 (oxalacetate)	0.073 (aspartate)		0.088
0.087	10 (pyruvate)	0.072 (alanine)		0.083

* The method for the determination of amino acids gave a standard deviation of $\pm$ 9%.

TABLE IV

REACTION BETWEEN ENZYME–PLP (0.38%) AND 0.02 *M*L–ALANINE IN 0.09 *M* PYROPHOSPHATE BUFFER (PH 8.4)

Absorbances of the reaction mixture	*Time after addition of alanine*						
	0 sec	*120 sec*	*240 sec*	*360 sec*	*480 sec*	*600 sec*	*5,000 sec*
360 mμ	0.530	0.508	0.466	0.406	0.356	0.328	0.260
335 mμ	0.325	0.338	0.360	0.389	0.415	0.430	0.460

to the number of coenzyme molecules bound to the enzyme. The determination of vitamin B_6-derivatives extracted from the enzyme revealed the presence of PLP and of traces only of PMP. The coenzyme/protein and activity/protein ratios of the enzyme isolated from the reaction mixture correspond to those found for enzyme–PLP.

As Table III shows, enzyme–PMP can react with pyruvate to give enzyme–PLP and alanine. This reaction is not immediate; it requires several minutes to reach completion. These latter results, together with those previously reported for the reaction of enzyme–PLP with alanine, suggest that GAT from pig heart is itself active also on alanine and pyruvate; the reaction of the enzyme with these substrates is much slower than the one with glutamate, aspartate, ketoglutarate or oxalacetate.

Taken as a whole, the results here described provide further evidence in support of the two steps by which enzymic transamination is postulated to occur.

ADDENDUM

When this manuscript was ready for publication, a paper on the same subject by T. W. JENKINS *et al.*[22] appeared. Our investigation of the reaction of GAT with each of its substrates gave results that are in substantial agreement with those obtained by these American authors on a different enzyme preparation and with different methods.

The results here described, moreover, provide original information about the reaction of the enzyme with non-substrate amino acids and with pyruvic acid; and these contribute to our knowledge of the specificity of the active site of the enzyme.

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