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PROPERTIES OF TAURINE : α-KETOGLUTARATE AMINOTRANSFERASE OF *ACHROMOBACTER SUPERFICIALIS*

INACTIVATION AND REACTIVATION OF ENZYME

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Summary

The activity of taurine : α-ketoglutarate aminotransferase (taurine : 2-oxoglutarate aminotransferase, EC 2.6.1.55) from *Achromobacter superficialis* is significantly diminished by treatment of the enzyme with $(NH_4)_2SO_4$ in the course of purification, and recovered by incubation with pyridoxal phosphate at high temperatures such as 60°C. The inactive form of enzyme absorbing at 280 and 345 nm contains 3 mol of pyridoxal phosphate per mol. The activated enzyme contains additional 1 mol of pyridoxal phosphate with a maximum at 430 nm. This peak is shifted to about 400 nm as a shoulder by dialysis of the enzyme, but the activity is not influenced. The inactive form is regarded as a partially resolved form, i.e. a semiapoenzyme. The enzyme catalyzes transamination of various ω-amino acids with α-ketoglutarate, which is the exclusive amino acceptor. Hypotaurine, DL-β-aminoisobutyrate, β-alanine and taurine are the preferred amino donors. The apparent Michaelis constants are as follows; taurine 12 mM, hypotaurine 16 mM, DL-β-aminoisobutyrate 11 mM, β-alanine 17 mM, α-ketoglutarate 11 mM and pyridoxal phosphate 5 μM.

Introduction

Taurine : α-ketoglutarate aminotransferase (taurine : 2-oxoglutarate aminotransferase, EC 2.6.1.55) catalyzes the transamination of taurine with α-ketoglutarate to yield sulfoacetaldehyde and L-glutamate [1]. We have purified the enzyme to homogeneity and crystallized it from *Achromobacter superficialis*

[2]. In this paper we describe the detailed properties of the enzyme, with emphasis on the inactivation and reactivation of it.

Materials and Methods

Materials. Pyridoxal-*P* and pyridoxamine-*P*, which were obtained from Kyowa Hakko Kogyo, Tokyo, and Sigma, were chromatographically purified by the procedure of Peterson and Sober [3]. Taurine aminotransferase was purified from *A. superficialis* (IRC B-0890) [2]. The enzyme was crystallized with $(NH_4)_2SO_4$. The crystals took the form of hexagonal plates.

Enzyme assay. The enzyme activity was determined, unless otherwise specified, after the enzyme was activated by preincubation with 0.1 mM pyridoxal-*P* at 60°C for 10 min.

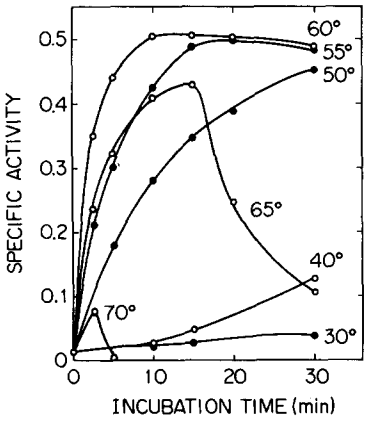
Method A. The reaction mixture was incubated and the activity was determined by measuring glutamate formed as described previously [2].

Method B. Sulfoacetaldehyde produced from taurine in the reaction mixture [2] was converted into the 2,4-dinitrophenylhydrazone, and separated from the unreacted reagents and the hydrazone of α-ketoglutarate as follows. To the supernatant solution (0.5 ml) was added 0.5 ml of 0.5% 2,4-dinitrophenylhydrazine solution in 2 M HCl, followed by incubation at 37°C for 30 min. Ethyl acetate (2 ml) was added to the solution and the mixture was shaken for 2 min. To a 1-ml aliquot of the aqueous layer was added 2 ml of a mixture of 1 M NaOH and 0.5 M Na_2CO_3 (1 : 1, v/v). After mixing the absorbance was measured against a blank at 435 nm within 3 min.

Protein determination. Protein was determined by measurement of absorbance at 280 nm ($A^{1\%}_{1cm} = 7.20$) [2].

Results

Activation of enzyme. In the course of purification, treatments of the enzyme with $(NH_4)_2SO_4$ ofted led to a significant loss of the activity as described previously [2]. The inactivated enzyme, which also was crystallized in the same form as the active one and pale yellow in color, exhibited only a little activity even in the presence of pyridoxal-*P*, but was found to be highly reactivated by preincubation with the coenzyme at high temperatures. The enzyme solution dialyzed against 10 mM potassium phosphate (pH 7.0) was incubated with 0.1 mM pyridoxal-*P* at various temperatures and the activity was determined under the standard conditions. The degree of activation is substantially dependent upon the preincubation temperature and time as shown in Fig. 1. The treatment with pyridoxal-*P* below 40°C caused only a slight activation, while the extent of activation was markedly enhanced with an increase in temperature up to 60°C. The maximum activation was observed when the enzyme was preincubated at 60°C for 10 min, or at 55°C for 20 min. The enzyme thus activated was stable at least for another 15—20 min at these temperatures in the presence of pyridoxal-*P*, and was used throughout for investigation of the properties of enzyme, unless otherwise stated. The activity decreased considerably rapidly, when the activated enzyme was kept at 60°C in the absence of the coenzyme. The treatment above 65°C caused irreversible



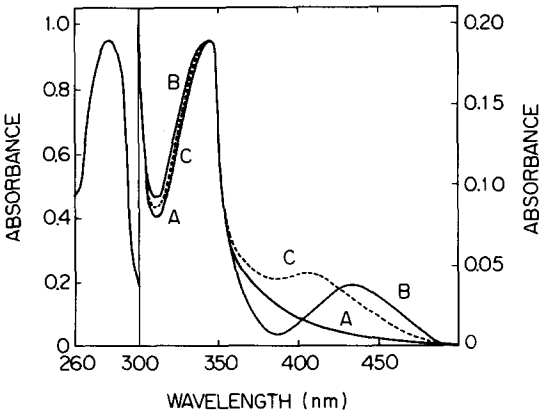


Fig. 1. Activation of the enzyme by incubation with pyridoxal-*P*. The inactivated enzyme was incubated with 0.1 mM pyridoxal-*P* at the indicated temperatures and the activity was determined by method B.

Fig. 2. Absorption spectra of the inactivated, reactivated and aged enzymes. (A), The inactivated enzyme (13 mg/ml) was dissolved in 0.01 M potassium phosphate (pH 7.0). (B), The enzyme (A) was incubated with 0.1 mM pyridoxal-*P* at 60°C for 10 min and the spectrum was taken against a buffer blank containing 0.1 mM pyridoxal-*P*. (C), The enzyme (B) was dialyzed at 4°C against 3 changes of 200 volumes of 0.01 M potassium phosphate (pH 7.0).

inactivation under the conditions employed. The inactivated crystalline enzyme, after dialysis, was preincubated at 60°C for 10 min with various concentrations of pyridoxal-*P*, followed by determination of the activity. The activity was increased with increasing concentration of pyridoxal-*P* and the maximum activity was obtained with 0.1 mM pyridoxal-*P*, though no activation was observed in the absence of pyridoxal-*P*. The activation effects of some other vitamin B_6 compounds were also investigated at 60°C. Pyridoxamine-*P* was as effective an activator as pyridoxal-*P* at 1 mM, and approximately 65% effective at 0.1 mM. Neither pyridoxine, pyridoxal, pyridoxamine nor pyridoxine-*P* activated the enzyme.

Spectrophotometric behavior and vitamin B_6 content. The absorption spectrum of the inactivated enzyme has maxima at 280 and 345 nm (Fig. 2, curve A); the molar absorption coefficients are 112 000 and 21 000, respectively on the basis of molecular weight of 156 000. The spectrum of the enzyme activated fully as described above exhibits a small new peak at 430 nm in addition to the peaks at 280 and 345 nm (Fig. 2, curve B). The spectrum is not affected by varying the pH (6.0—9.0). The absorption at 430 nm increased with increase of the preincubation time up to 10 min (at 60°C), when the activation reached a maximum. The 430-nm peak was not formed by preincubation without pyridoxal-*P*. The exhaustive dialysis of the activated enzyme against 0.01 M potassium phosphate, pH 7.0 (e.g. for 40 h at 4°C) resulted in disappearance of the 430-nm peak and concomitant formation of a shoulder at about 400 nm (Fig. 2, curve C), though the activity was not influenced. When the dialyzed enzyme was incubated again with 0.1 mM pyridoxal-*P* at 60°C for 10 min, the peak at 430 nm did not reappear.

The vitamin B_6 contents of the inactivated and activated enzymes were determined by the microbiological assay with *Saccharomyces carlsbergensis*

[4,5] to be 2.8 and 3.9 mol per 156 000 g of protein, respectively. Difference in molecular weight (approximately 156 000 [2]) between both the forms of enzyme was not observed.

Resolution and reconstitution of enzyme. Pyridoxal-*P* was not required for the maximum activity of the activated enzyme even after dialysis or Sephadex G-25 gel filtration. A decrease in absorbance at about 400 nm was caused by addition of taurine or β-alanine to the activated enzyme, but the 345-nm peak was not practically affected. The enzyme was incubated with 50 mM taurine in 0.01 M potassium phosphate (pH 7.0) at 30°C for 1 h, followed by dialysis against two changes of the buffer. The enzyme treated with taurine did not exhibit a shoulder at about 400 nm at all, but was catalytically active in the absence of added pyridoxal-*P*. These results suggest that the bound pyridoxal-*P* of the enzyme reacts with taurine to form the pyridoxamine-*P* form of enzyme, which is catalytically active, and that the bound pyridoxamine-*P* cannot be released by dialysis. An attempt was made to resolve the cofactor from the enzyme. The enzyme treated with taurine was incubated with 0.5 M potassium phosphate (pH 5.0) at room temperature for 1 h, followed by precipitation with $(NH_4)_2SO_4$ (60% saturation). The precipitate was dissolved in a small volume of 0.01 M potassium phosphate (pH 7.0), and dialyzed overnight against the same buffer. The enzyme had almost no detectable activity in the absence or presence of added pyridoxal-*P*, although the full activity was obtained after incubation with pyridoxal-*P* at 60°C for 10 min. The inactive form of enzyme did not exhibit a absorption peak at about 400 nm, but did one at 345 nm. The active enzyme reconstituted with pyridoxal-*P* showed a shoulder at about 400 nm. Thus, pyridoxal-*P* or pyridoxamine-*P* tightly bound to the enzyme is released by the treatments. The resolved enzyme can be reconstituted by incubation with either pyridoxal-*P* or pyridoxamine-*P* at approximately 60°C for 10 min as described above.

Stability of enzyme. Both the inactivated and activated enzymes can be stored at 4°C as a suspension in 10 mM potassium phosphate (pH 7.0) containing 0.1 mM pyridoxal-*P* and 60% saturated $(NH_4)_2SO_4$ with little loss of activity, and as a solution in the above-mentioned buffer containing only the cofactor for periods of over 5 and 2 months, respectively. Deep freezing and thawing led to the significant inactivation of the enzyme. The presence of pyridoxal-*P* protected the activated enzyme from inactivation even when the enzyme was kept at 60°C as described above.

Effect of pH. The enzyme is active in the alkaline pH region with a maximum at 7.8—8.0. The effect of pH on the stability of the enzyme also investigated. When the enzyme was heated at 60°C in 50 mM buffers of various pH for 10 min, the enzyme was found to be stable between pH 6.0 and 9.0.

Substrate specificity. Hypotaurine (relative activity, 601), DL-β-aminoisobutyrate (208) and β-alanine (184) showed higher reactivity than taurine (100) in the transamination. In addition to them β-aminobutyrate (60), 3-aminopropanesulfonate (43) and DL-β-aminobutyrate (14) also serve as amino donors, whereas aminomethanesulfonate, glycine, δ-aminovalerate, L- and D-α-amino acids including alanine and lysine, and amines are not substrates. We showed that sulfinoacetaldehyde is produced from hypotaurine, and converted spontaneously into acetaldehyde and SO_2 [6]. α-Ketoglutarate is the exclusive

TABLE I

EFFECT OF INHIBITORS ON ENZYME ACTIVITY

The activity was determined by method B.

Inhibitors	Concentration (mM)	Relative activity
None	—	100
Semicarbazide · HCl	0.5	79
NH_2OH · HCl	0.5	0
L-Penicillamine	0.5	100
D-Penicillamine	0.5	100
L-Cycloserine	0.5	3
D-Cycloserine	0.5	80
MBTH *	0.5	88
Phenylhydrazine · HCl	0.5	83
P-Chloromercuribenzoate	0.1	100
Iodoacetate	0.5	100
α,α'-Dipyridyl	0.5	100
Ethylenediaminetetraacetate	0.5	100
$HgCl_2$	0.1	2
$CuSO_4$	0.5	100
$FeCl_3$	0.5	100

* 3-Methyl-2-benzothiazolone hydrazone hydrochloride.

amino acceptor for the amino donors; pyruvate, phenylpyruvate and oxaloacetate are inert. L-Glutamate was formed exclusively from α-ketoglutarate even when DL-β-aminoisobutyrate was used as an amino donor.

Kinetics. The apparent Michaelis constants were calculated: taurine 12 mM, hypotaurine 16 mM, DL-β-aminoisobutyrate 11 mM, β-alanine 17 mM and α-ketoglutarate 11 mM. The apparent K_m value of about 5 μM also was obtained for pyridoxal-*P*, when taurine was used.

Effect of inhibitors. NH_2OH and L-cycloserine strongly inhibit the enzyme, while semicarbazide, D-cycloserine and a few other carbonyl reagents are weak inhibitors (Table I). The absorption spectrum and reactivation of the enzyme inactivated by NH_2OH were examined. To a solution of the activated and dialyzed enzyme, NH_2OH was added to give a final concentration of 0.5 mM followed by incubation at 30°C for 60 min. The solution was dialyzed against 10 mM potassium phosphate (pH 7.0). The shoulder in the region of 400 nm of the activated and dialyzed enzyme disappeared, whereas the peak at 345 nm did not change significantly by the NH_2OH treatment. An attempt was made to reactivate the NH_2OH-inactivated enzyme with pyridoxal-*P*. The NH_2OH-inactivated enzyme was incubated at 60°C for various periods in the absence or presence of 0.1 mM pyridoxal-*P*, and the activity was determined. The enzyme was reactivated by incubation with pyridoxal-*P*, and the reactivation depended upon the incubation time. The enzyme was only slightly reactivated when incubated without pyridoxal-*P*. The enzyme thus reactivated exhibits a shoulder in the region of 400 nm.

Discussion

We found here a loss of the enzyme activity by treatment with $(NH_4)_2SO_4$, and reactivation by incubation with pyridoxal-*P* at high temperatures. DeVivo

and Peterkofsky [7] observed the heat activation of tyrosine aminotransferase with pyridoxal-*P* and α-ketoglutarate. Recently Shioji et al. [8] also reported the similar observation. The inactivated (λ_{max} 345 nm) and reactivated (λ_{max} 345 and 430 nm) forms of taurine aminotransferase contain 3 and 4 mol of vitamin B_6 per mol, respectively. The inactive enzyme is probably identical with the inactive form resolved by incubation with taurine in view of their absorption spectra and coenzyme contents. Thus, 1 mol of the bound pyridoxal-*P* absorbing at 400—430 nm is released from the protein by $(NH_4)_2SO_4$ treatment or incubation with taurine and a high concentration of phosphate (pH 5.0), and regained by incubation with added pyridoxal-*P* at high temperatures. Pyridoxal-*P* absorbing at about 400 or 430 nm is firmly bound to the protein moiety in an active (holo-) form. The resolved enzyme cannot bind with the coenzyme at physiological temperatures, but heating the enzyme favors its reconstitution. The catalytic activity of the enzyme is concerned exclusively with this 400 or 430 nm bound pyridoxal-*P*, which is converted into pyridoxamine-*P* by incubation with the amino donor. The catalytically inactive form with an absorption maximum at 345 nm is regarded as a kind of "semiapoenzyme" as reported by us for L-lysine aminotransferase [9] and D-amino acid aminotransferase [10]. The semiapo forms of these enzymes are half resolved, whereas in the semiapoenzyme of taurine aminotransferase only a quarter of the bound coenzyme is dissociated. Though the function of the 345-nm pyridoxal-*P* in the enzyme action has not yet been elucidated, the pyridoxal-*P* is not at least directly involved in the catalytic process. The 430-nm peak of the activated enzyme, by analogy with other aminotransferases, probably is ascribed to an aldimine linkage between pyridoxal-*P* and an amino group of lysine residue of the protein. The peak is shifted apparently to about 400 nm by dialysis. Both the 430- and 400-nm forms of enzyme are catalytically active. Although this spectral shift has not been elucidated, it is probable that the structure of the coenzyme-binding site and its vicinity, or the mode of binding of the coenzyme is changed by aging the enzyme in the substantially absence of added pyridoxal-*P* to affect the absorption spectrum.

The results obtained on the susbtrate specificity show that the amino donor must be a neutral ω-amino acids with the amino groups being separated by from 2 to 3 carbon atoms. With regard to this the enzyme is similar to β-aminoisobutyrate aminotranferase of mouse brain [11], but they are different in the relative activity for the amino donors. β-Aminoisobutyrate aminotransferase of hog kidney catalyzes the transamination of L-β-aminoisobutyrate, β-alanine and γ-aminobutyrate, but taurine is not a substrate [12]. γ-Aminobutyrate aminotransferase of *Pseudomonas fluorescens* exclusively transaminates γ-aminobutyrate [13], and the enzyme of *Bacillus cereus* catalyzes transamination of γ-aminobutyrate, δ-aminovalerate and ε-aminocaproate [14]. Taurine also is transaminated by this enzyme, though very slowly. These findings show the diversity of ω-amino acid aminotransferases and of their functions in the organisms.

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