Kinetic Characteristics, Substrate Specificity and Catalytic Properties of Phosphoserine Aminotransferase from the Green Alga *Scenedesmus obliquus*, Mutant C-2 A′

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Scenedesmus obliquus, Phosphoserine aminotransferase (PSAT, E.C. 2.6.1.52), Phosphorylated Pathway, DOVA and GSA as Substrates.

Phosphoserine aminotransferase (EC 2.6.1.52) has been purified from *Scenedesmus obliquus*, mutant C-2A′, as reported previously (Stolz and Dörnemann, 1994). The current studies on its catalytic properties, involving initial reaction velocities as a function of the phosphoserine concentration at various fixed concentrations of 2-oxoglutarate as amino-acceptor, indicate a bi-bi ping pong mechanism. The application of a variety of substrate analogues of phosphoserine revealed no significant metabolisation of these compounds and thus a considerable specificity of the enzyme. 4,5-dioxovalerate with glutamate as aminodonor is effective as competitive substrate to phosphohydroxypyruvate in the forward reaction and yields 5-aminolevulinate.

4,5-Dioxovalerate and glutamate-1-semialdehyde can both serve as competitive aminoacceptor in the reverse reaction with phosphoserine and as substrate with 2-oxoglutarate as aminoacceptor. Comparison of the phosphoserine transamination with the transamination of 4,5-dioxovalerate revealed for both reactions a pH-optimum of 6.8–7.0 in Mes/Bis-Tris-buffer. However, the K_m-values and the V_{max} for phosphoserine and 2-oxoglutarate on the one side, and 4,5-dioxovalerate and glutamate on the other were found to differ by orders of magnitude.

Introduction

Phosphoserine aminotransferase is one of the enzymes of the "phosphorylated pathway", leading to the formation of serine from 3-phosphoglycerate. The glycolic intermediate D-3-phosphoglycerate is oxidized by D-3-phosphoglycerate dehydrogenase (EC 1.1.1.95) to form phosphohydroxypyruvate (Eqn. (1)). The penultimate step of this pathway, the transamination of phosphohydroxypyruvate with glutamate as an aminodonor to yield phosphoserine (Eqn. (2)) is catalyzed by phosphoserine aminotransferase (PSAT, EC 2.6.1.52). The final step of the reaction sequence is the dephosphorylation of 3-phosphoserine (Eqn. (3)) by phosphoserine phosphatase (EC 3.1.3.3) (Ichihara and Greenberg, 1957; Walsh and Sallach, 1966). The reaction sequence is summarized below in Eqn. (1–3):

$$\text{3-phosphoglycerate} + NAD^+ = \text{3-phosphohydroxypyruvate} + NADH \quad (1)$$

$$\text{3-O-phosphohydroxypyruvate} + \text{L-glutamate} = \text{O-phospho-L-serine} + \text{2-oxoglutarate} \quad (2)$$

$$\text{L-(or D)-O-phosphoserine} + H_2O = \text{L-(or D)-serine} + \text{orthophosphate} \quad (3)$$

PSAT has been isolated from different animal tissues (e.g. bovine liver, Lund *et al.*, 1987; sheep brain, Hirsch and Greenberg, 1967), as well as from bacterial (*E. coli*, Lewendon, 1984; *Rhodopseudomonas capsulata*, Schmidt and Sojka, 1973) and plant organisms (pea, Cheung *et al.*, 1968, Walton and Woolhouse, 1986; spinach, Larsson and Albertson, 1979; soybean root nodules, Reynolds *et al.*, 1986 and 1988). The characterization of PSAT from different tissues reveals, that the enzymatic reaction is an equilibrium reaction

Abbreviations: ALA, 5-aminolevulinate; Bis-Tris, 2,2, bis-(hydroxymethyl)-2,2′,2″-nitrilotriethanol; Glu, glutamate; GSA, glutamate-1-semialdehyde; MES, 2ß-morpholino-ethanesulfonic acid; OG, 2-oxoglutarate; P-ser, *o*-phospho-L-serine; PSAT, *o*-phospho-L-serine: 2-oxoglutarate aminotransferase; Tris, tris-(hydroxymethyl)-aminomethane.

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(Merrill *et al.*, 1981,) and follows a bi-bi ping pong mechanism (Basurko *et al.* 1989). Reports about the pH-optima of the different enzymes vary between 6.8 and 8.2 (Basurko *et al.*, 1989; Walsh and Salach, 1966; Hirsch and Greenberg, 1967; Kallen *et al.*, 1987).

Experiments on the substrate specificity of phosphoserine aminotransferase for a variety of amino acids and DL-aminophosphonates only revealed moderate metabolization of DL-phosphothreonine (Lund *et al.*, 1986), homocysteate, 2-amino-4-phosphonobutyrate and 2-amino-5-phosphonovalerate (Basurko *et al.*, 1989) by PSAT. As a conclusion, PSAT is considered as an enzyme with a quite high specificity for its substrate.

In a previous paper we have shown the purification of PSAT from the green alga *Scenedesmus obliquus*, mutant C-2A′ (Stolz and Dörnemann, 1994). In the current publication we will present kinetic properties and catalytic characteristics of PSAT, as well as data on the competition of intermediates of the C_5-pathway to chlorophyll with phosphoserine on the substrate and amino acceptor/donor level of the enzyme.

Materials and Methods

Chemicals

L-lactate dehydrogenase and D-3-phosphoglycerate dehydrogenase were purchased from Sigma (Deisenhofen, F.R.G). All other applied chemicals and buffer components were p.a. grade or of the highest available purity. DOVA was prepared as described earlier (Dörnemann and Senger, 1980). The synthesis of GSA will be described in a forthcoming paper or may be performed as described by Gough *et al.* (1989).

Plant material and growth conditions

For all experiments the X-ray induced pigment mutant C-2A′ (Bishop, 1971) of *Scenedesmus* was used. In contrast to the wild type, the mutant synthesizes chlorophyll only in the light. After heterotrophic growth in darkness for 3–4 days (Bishop and Senger, 1972) the algae were exposed to white light of an intensity of 20 $W \times m^{-2}$ for 6 h.

Origin of the applied purified phosphoserine aminotransferase preparations

For the kinetic studies on PSAT fractions from the DE 52-cellulose- and Fractogel TSK DEAE-650-purification steps were used. For the demonstration of DOVA-transaminating activity of PSAT with glutamate as aminodonor to form ALA the homogeneous enzyme from a Mono Q-preparation was applied. For details concerning the different purification steps of PSAT to final apparent homogeneity see Stolz and Dörnemann (1994).

Assay for the transamination of phosphoserine

PSAT activity was determined spectrophotometrically applying a modification of the coupled assay procedure according to Hurst *et al.* (1981). The reaction mixture was 50 mM in Tris and 25 mM in Bis-Tris, pH 8, containing additionally 1 mM DTT, 2.5 mM L-phosphoserine, 0.12 mM NADH, 3 mU of L-lactate dehydrogenase and 0.5 U of D-3-phoshoglycerate dehydrogenase in a final volume of 1 ml. The reaction mixture was preincubated at 30 °C for 15 min and the transamination reaction initiated by the addition of a PSAT protein sample. The reaction was followed by the measurement of NADH consumption at 340 nm, for calculations using the molar extinction coefficient of 6.22 $mM^{-1} \times cm^{-1}$ at 340 nm.

Assay for the transamination of DOVA to ALA

Following the modified procedure of Kah *et al.* (1988) the transamination of DOVA to ALA with glutamate as aminodonor was tested in 50 mM MES/50 mM Bis-Tris-buffer, pH 6.8, which additionally contained 1 mM DTT, 25 mM glutamate and 0.1 mM levulinic acid (LA). LA is a competitive inhibitor of ALA-dehydratase and was added to prevent a possible further metabolization of ALA to porphobilinogen. The reaction mixture (300 µl) was incubated at 30 °C for 30 min and the reaction stopped by adding 5 µl of 70% perchloric acid. The assay mixture was then adjusted to pH 6.9 with a saturated solution of sodium phosphate and the accumulated ALA reacted with 100 µl of ethylacetoacetate for 20 min at 100 °C to form 2-methyl-3-carbethoxy-4(3′-propionic acid)-pyrrole (ALA-pyrrole). The pyrrole was quantified with

Ehrlich's reagent as described by Mauzerall and Granick (1956). For calculation the molar extinction coefficient of $7.2\times10^4\times M^{-1}\times cm^{-1}$ at 553 nm was used.

Studies on the substrate specificity of PSAT

The substrate specificity of PSAT was tested employing a variety of amino acids and aminophosphonates, which are, concerning their reacting group, similar to the substrate (Basurko *et al.*, 1989). The following analogues were tested: cystein, homocystein, serine phosphotyrosine, phosphothreonine, 2-amino-4-phosphonobutyrate, 2-amino-5-phosphonovalerate. As in preliminary experiments DOVA also showed some reaction we also applied intermediates of the C_5-pathway to chlorophyll: GSA, DOVA, DAVA and ALA. PSAT-activity was determined by replacing the amino donor (1 mM phosphoserine) or the amino acceptor (1 mM oxoglutarate) by 1 mM substrate analogue, respectively. Values were calculated on the basis of the enzyme activity of the controls with 1 mM phosphoserine and 1 mM 2-oxoglutarate (100%).

Comparative kinetic studies on the transamination of 4,5-dioxovalerate and phosphoserine

Comparative kinetic studies of both transamination reactions were carried out in 50 mM MES/50 mM Tris-buffer as described above. Concentrations of phosphoserine, 2-oxoglutarate, DOVA and glutamate were varied as indicated in the Results section. The pH-optima of the transamination reactions were determined using 50 mM Mes/50 mM Bis-Tris- and 50 mM Mes/50mM Tris-buffer, respectively.

Studies on the pH-dependence of V_{max}

For the studies on the pH-dependence of V_{max} a highly purified preparation of PSAT from the Fractogel-TSK 650 DEAE-purification step was used. V_{max} was tested at different pH-values ranging from pH 6.5 to pH 8.5 in 25 mM Bis-Tris/50 mM Tris-buffer. Varying phosphoserine concentrations (5–60 μM) were applied at a fixed 2-oxoglutarate concentration of 17.5 mM and vice versa, varying 2-oxoglutarate concentrations (50–600 μM) at a fixed phosphoserine concentration of 2.5 mM.

Studies on the reaction mechanism of PSAT

For the studies on the initial reaction velocities of PSAT a highly purified preparation from the Fractogel-TSK 650 DEAE-purification step was used. PSAT activity was tested with increasing phosphoserine concentrations (0.016–1mM) and at various fixed concentrations of 2-oxoglutarate (0.01 mM, 0.1 mM, 1 mM).

Results and Discussion

Substrate specificity of PSAT and side reactions

PSAT from various animal, bacterial and plant sources was shown to be quite specific for its substrate phosphoserine (Lund *et al.*, 1987, Basurko *et al.*, 1989). We thus made attempts to verify this finding also for the purified enzyme from *Scenedesmus*.

To study the substrate specificity of PSAT a variety of substrate analogues of phosphoserine like serine, cysteine, homocysteine, phosphotyrosine, phosphothreonine, 2-amino-4-phosphonobutyrate and 2-amino-5-phosphonovalerate were tested in the reverse reaction of the protein. The compounds were applied in 1 mM concentration, replacing phosphoserine. None of the analogues revealed more than 2.5% of the maximum reaction with phosphoserine indicating that PSAT is quite highly specific for its substrate.

During the purification of the enzyme it turned out that PSAT also shows some affinity to 4,5-dioxovalerate (DOVA). To support this finding homogeneous PSAT from a Mono Q-preparation was tested with DOVA and glutamate for DOVA-transaminase activity in a variation of the assay employed by Kah *et al.* (1988). The formed ALA was condensed with ethylacetoacetate to ALA-pyrrole and the pyrrole concentration determined by the addition of Ehrlich's reagent. Fig. 1 represents the absorption spectrum of the ALA-pyrrole derived from DOVA, which was transaminated by homogeneous PSAT. The control was taken with denatured enzyme. By employing highly purified PSAT we could exclude reactions of contaminating enzyme activities, which is important, as in *Scenedesmus* the existence of L-glutamate: 4,5-dioxovalerate aminotransferase could be shown (Kah *et al.*, 1988). Furthermore, the transamination of DOVA with glutamate or alanine as amino

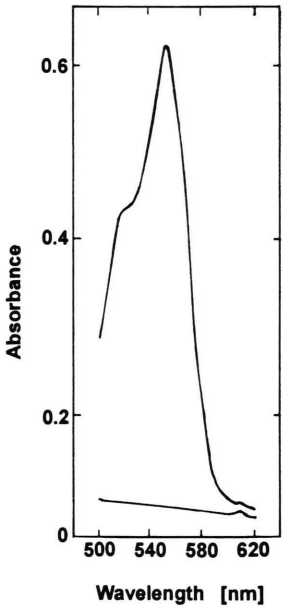

Fig. 1. Absorption spectrum of the ALA-pyrrole-Ehrlich-complex of ALA formed by PSAT from DOVA and the respective control with denatured protein.

donors to ALA can be performed by alanine:glyoxylate aminotransferase as shown by Nogushi and Mori (1981). This reaction presumably represents a side-reaction of the enzyme. Such side reactions are, however, quite usual for aminotransferases (Christen and Metzler, 1985).

To elucidate whether DOVA, as well as other intermediates of the C_5-pathway, DAVA, GSA or ALA, can serve as substrates of the main transamination reaction of PSAT, their roles as substrate analogues were tested as described above. When phosphoserine was replaced as amino donor, only with GSA a reaction of 20% of the control could be measured. When 2-oxoglutarate was substituted as amino acceptor by either GSA or DOVA, GSA was effective with 7.4% of the maximum enzyme activity and DOVA with 7%. Thus it is shown, that two of the intermediates of the C_5-pathway, GSA and DOVA, can serve as substrate to PSAT, forming ALA. This side reaction of PSAT appears to be reasonable, as the structure of the reacting groups of DOVA and GSA are very similar to the reacting group of phosphohydroxypyruvate and phosphoserine, respectively. The aldehyde groups of the analogues are obviously recognized as carboxyl groups.

Determination of the pH-optimum and the K_m-values of PSAT

The pH-optimum of *Scenedesmus*-PSAT varries between pH 6.8 and 8.2, depending on the applied buffer system (Mes/Bis-Tris: 6.9–7; Tris: 7.6; Bis-Tris/Tris: ≈8). This phenomenon is quite common. For PSAT pH-optima between pH 6.8 and pH 8.2 were determined in different buffers (beef liver-PSAT in phosphate buffer: pH 6.8–7.2, Basurko *et al.*, 1988; *E. coli*-PSAT: 7.5–8.5, Kallen *et al.*, 1987; PSAT of soybean root nodules in citrate/glycine/K-phosphate-buffer: pH 7.5–9, Reynolds, 1988; sheep brain in Tris/ammonium-acetate-buffer: pH 8.15, Hirsch and Greenberg, 1966). Under our assay conditions the highest enzyme activity of the *Scenedesmus*-enzyme was reached with Bis-Tris/Tris-buffer at pH 8.

To compare the pH-optimum of phosphoserine-transamination with that of DOVA-transamination we determined the enzyme activity in a mixture of 50 mM Mes- and 50 mM Bis-Tris-buffer. Between pH 6 and 8 the enzyme exhibits 75–100% activity with an optimum around pH 7 for phosphoserine with 2-oxoglutarate as aminoacceptor (Fig. 2).

The pH-dependence of the side reaction of PSAT with DOVA as substrate and glutamate as aminodonor to form ALA was determined employing a variation of the DOVA-transaminase as-

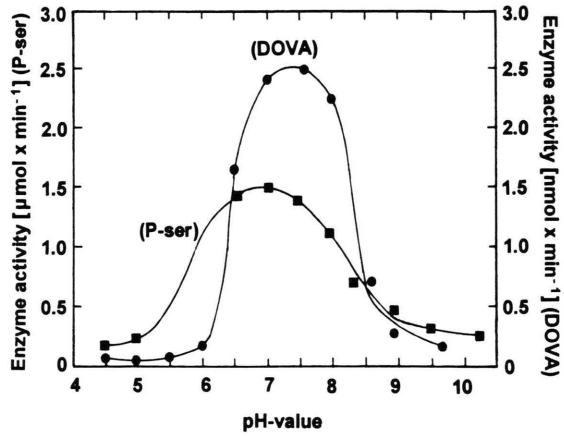

Fig. 2. Effect of the pH on PSAT from *Scenedesmus obliquus* with phosphoserine (■) and DOVA (●) as substrates and 2-oxoglutarate and glutamate, respectively, as amino acceptor/donor. Experimental conditions are described in the text.

say of Kah *et al.* (1988). The shape of the resulting curve was similar to that of the main reaction, exhibiting 80–100% activity between pH 6.5 and 8.2 with a plateau of optimum activity between pH 7 and 8 (Fig. 2). It has, however, to be noted that the conversion of DOVA is only 0.1% of that of phosphoserine, clearly discriminating DOVA metabolization as a side reaction of the enzyme.

K_m-values and V_{max} of phosphoserine- and DOVA-transamination

Identical conclusions can be drawn from the determinations of the K_m-values and the maximum reaction velocities (V_{max}) of both, the substrates of the transamination reactions, phosphoserine and DOVA, and the amino acceptor/donor, 2-oxoglutarate and glutamate (Fig. 3). The K_m-value of PSAT for phosphoserine at a fixed 2-oxoglutarate concentration of 17.5 mM was determined to be 83±4 μM, V_{max} beeing 1.33±0.1 μmol×min^{-1} under these conditions. Evaluation of the data by Lineweaver-Burk plots resulted in values of 84±4 μM and 1.64±μmol×min^{-1}. For DOVA at a fixed glutamate concentration of 10 mM the K_m-value turned out to be 840±20 μM at a V_{max} of 6.4±0.5 nmol×min^{-1} (Fig. 3a) (Lineweaver-Burk plot: K_m= 910±20μM, V_{max}=5.56±0.5 nmol×min^{-1}). This means that the affinity of PSAT to the two substrates differs by a factor of ten and thus that DOVA-transamination is only a side reaction. This interpretation is supported by the fact that the corresponding maximum reaction velocities even differ by a factor of about 250 in favour of phosphoserine. Furthermore, it turned out that phosphoserine- and DOVA-concentrations above 2.5 mM act inhibitory on the enzyme.

The K_m-value for the amino acceptor of phosphoserine transamination, 2-oxoglutarate, was determined to be 180±10 μM at a fixed phosphoserine concentration of 2.5 mM, for the amino donor of DOVA-transamination, glutamate (fixed concentration 10 mM), 233±20 μM, using the Hanes-plot for evaluation of the data. The corresponding maximum reaction velocities were 0.96±0.1 μmol×min^{-1} and 3.15±0.3 nmol×min^{-1}. Results obtained by Lineweaver-Burk plots were: K_m 177±10μM and 244±20μM, V_{max} 0.87±0.1 μmol×min^{-1} and 4.33±0.4 nmol×min^{-1}. No inhibition by 2-oxoglutarate and glutamate was observed with concentrations up to 17,5 mM. Obviously, there is no significant difference in the affinity of PSAT to the amino acceptor/donor, which can be explained by the fact that glutamate and 2-oxoglutarate are in both cases the natural amino donor/acceptors. However, there is again a big difference in the corresponding maximum reaction velocities which differ by a factor of about 300 in favour of the main reaction. As an explanation for the latter phenomenon it may be speculated that DOVA is indeed quite specifically bound to the enzyme, but its transamination to ALA occurs very slowly with PSAT compared to the reaction of phosphoserine and 2-oxoglutarate to phosphohydroxypyruvate, as in the case of DOVA an aldehyde group compared to an oxo-

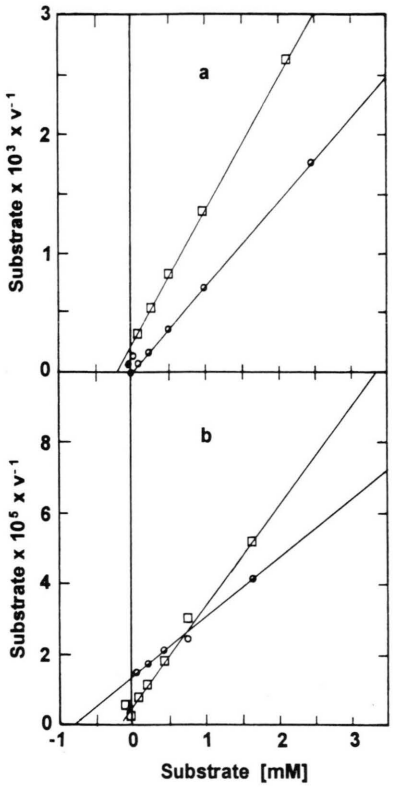

Fig. 3. Hanes kinetic plots. a) The transamination of phosphoserine (7.8 μM-2500 μM) at constant (17.5 mM) 2-oxoglutarate (○) and vice versa, of 2-oxoglutarate (137 μM-17500 μM) at a constant (2.5 mM) phosphoserine concentration (□). b) The transamination of DOVA (104 μM-1680 μM) by PSAT at constant (5 mM) glutamate (○) and, vice versa, of glutamate (15.6 μM-1000 μM) at a constant (2 mM) DOVA concentration (□).

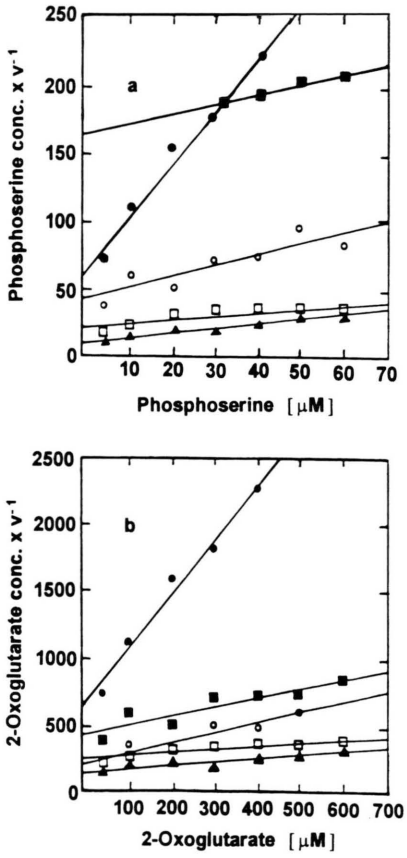

Fig. 4. Hanes kinetic plots of PSAT from *Scenedesmus obliquus* for the determination of the initial reaction velocities (V_{ini}) in dependence of the pH-value: pH 6.5: ●; 7.0: ■; 7.6: □; 8.0: ▲; 8.5: ○. a: variable P-ser concentration at 17.5 mM OG; b: variable OG at 2.5 mM P-ser.

group is transaminated. This might very well influence velocities of formation of the enzyme substrate complex and the dissociation of the enzyme product complex, resulting in a much slower velocity for this reaction. Compared to the reaction velocity of the transamination of DOVA with glutamate by glutamate:4,5-dioxovalerate aminotransferase from *Scenedesmus*, which was reported to be 37.2 nmol×min^{-1} (Kah *et al.*, 1988), the PSAT-reaction with DOVA is about one order of magnitude slower than with the specific enzyme, but the two reactions are much more comparable in their velocity than the reaction velocities of PSAT with phosphoserine and DOVA, respectively.

pH-dependance of V_{max} of PSAT

Furthermore, studies on the pH-dependence of V_{max} for both, phosphoserine and 2-oxoglutarate, were performed. Therefore, varying phosphoserine concentrations at a fixed 2-oxoglutarate concentration of 17.5 mM and, vice versa, varying 2-oxoglutarate concentrations at a fixed phosphoserine concentration of 2.5 mM were incubated at different pH-values between 6.5 and 8.5. The results for both series of experiments are shown in Fig. 4 a and b. The tangens of the slopes of the different curves is reciprocal to the initial reaction velocity at each pH-value (tanα=1/v) and represents thus a measure for the dependence of V_{max} from the pH-value. The data presented show that the variation of the apparent V_{max} as a function of the pH is sharply dependent on the proton concentration (Fig. 4a, b). This becomes even more evident when V_{max} is plotted against the pH (Fig. 5). It can be seen that the reaction velocity reaches its maximum arround pH 7.5, at pH 7 and 8 only beeing 32 and 76% of the maximum for phosphoserine, and 35 and 80% for oxoglutarate, respectively. It has thus to be stated that the V_{max} of PSAT is much more sensitive to changes in pH than the general enzyme activity which shows a broad maximum of 80 to 100% between pH 6.5 and 8.2.

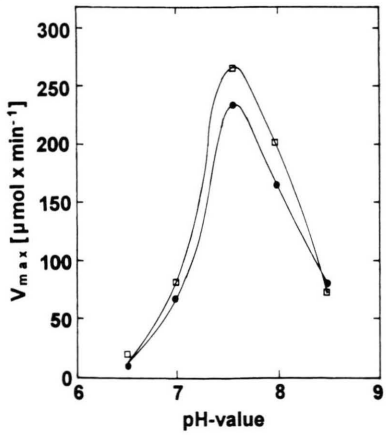

Fig. 5. Dependence of the V_{max} of *Scenedesmus*-PSAT on the pH. V_{max} was calculated from the initial reaction velocities given in Fig. 4 (tan α=1/v). V_{max} is expressed as μmol x min^{-1}. P-ser variable, OG fixed (●); OG variable, P-ser fixed (□).

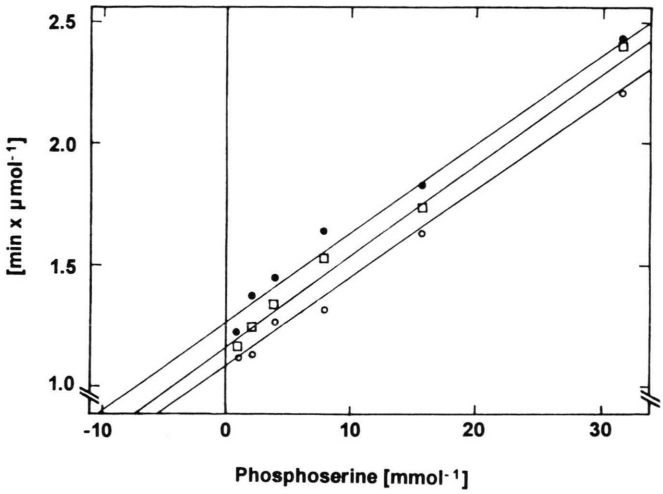

Fig. 6. Lineweaver-Burk-plot of the PSAT-reaction with varying P-ser concentrations at three fixed 2-OG concentrations of 1 mM: ○; 0.1 mM: □; 0.01 mM: ●. The apparently identical slopes point out a bi-bi ping pong mechanism of the transamination reaction.

Mechanism of the PSAT-reaction

Further studies were carried out on the transamination mechanism of PSAT. Therefore, three different concentrations of 2-oxoglutarate (0.01 mM, 0.1 mM, 1 mM) were incubated with varying concentrations of phosphoserine (0.016 to 1 mM). The obtained initial reaction velocities were plotted double-reciprocally against the 2-oxoglutarate concentration (1/v *versus* 1/conc. 2-oxoglutarate, Fig. 6). From the parallel steady-state pattern of the obtained curves it can clearly be concluded that also PSAT from *Scenedesmus* follows a bi-bi ping pong mechanism as proposed for the enzyme from beef liver (Basurko *et al.*, 1989).

Acknowledgements

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