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INTERACTIONS OF L-SERINE DEHYDRATASE FROM RAT LIVER WITH ITS COENZYME AND SUBSTRATES

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SUMMARY

With L-serine dehydratase (L-serine hydrolase (deaminating) EC 4.2.1.13) the ratio of the activities on L-threonine and on L-serine varies with the pyridoxal 5'-phosphate (PLP) and enzyme concentration if the enzyme is incubated with one of the substrates at a time. Incubating the enzyme with both substrates together, the T/S ratio remains constant. To explain this phenomenon, the PLP–enzyme dissociation constants were determined under various conditions.

K^+ and NH_4^+ decrease the PLP dissociation constants of the enzyme. Dilution of the enzyme increases the constant, probably due to the fact that the enzyme dissociates into its subunits which have a lower affinity for PLP.

Tris and the substrates compete for PLP with the enzyme because of Schiff's base formation.

It can be concluded that the T/S ratio varies because with the range in the PLP–enzyme dissociation constant, the enzyme is not always saturated with PLP, and the two substrates compete differently for PLP with the enzyme.

INTRODUCTION

After purifying L-serine (L-threonine) dehydratase from rat liver (L-serine hydrolase (deaminating) EC 4.2.1.13) to homogeneity, Nakagawa and Kimura [1] found that the enzyme degrades L-serine, as well as L-threonine. Studies from our laboratory [2] demonstrated that the ratio of the initial velocities for the reactions with L-threonine and L-serine (T/S ratio) is not constant under various conditions. Dilution of the enzyme in the absence of K^+ or NH_4^+ decreases the T/S ratio, whereas addition of K^+ or NH_4^+ to the assay mixture increases the activity on L-threonine more than that on L-serine. We had proposed that threonine and serine differently affect the binding of pyridoxal 5'-phosphate (PLP) to the apoenzyme, thus altering differently the balance between holo- and apoenzyme in the incubation mixture. As threonine is an essential amino acid in the rat, it was of interest to learn

Abbreviation: PLP, pyridoxal 5'-phosphate.

the mechanism of the change in the T/S ratio and whether it could be of physiological significance.

MATERIALS AND METHODS

Chemicals

L-Serine and L-threonine were obtained from Merck AG. PLP, dithiothreitol, and bovine serum albumin were supplied by Boehringer Mannheim GmbH. All other reagents were of analytical grade.

The enzyme

The enzyme was purified from 60 female Wistar rats (approx. 150 g) which had been fed a high protein diet for seven days to induce the enzyme. The purification procedure included an $(NH_4)_2SO_4$ fractionation from 28–45%, heat treatment (2.5 min at 55 °C), Sephadex G-100 filtration, and DEAE-cellulose gradient elution. The purified enzyme had a specific activity of about 16 000 units/mg protein and migrated as one band on disc electrophoresis. The recovery was 13%. For details see ref. 3.

Preparation of the apoenzyme

The native enzyme was dialysed for 36 h against 800 volumes of 0.1 M Tris–HCl buffer, pH 8.0, containing 1 mM EDTA, and 0.1 mM dithiothreitol with four buffer changes. Then the apoenzyme was equilibrated with the indicated buffer by 4-h dialysation or by passage through a Sephadex G-25 column.

Enzyme assay

The standard assay mixture contained in a total volume of 2.5 ml: 250 μmoles L-serine or L-threonine, 0.5 μmole PLP, 250 μmoles sodium pyrophosphate–HCl buffer, pH 8.4, or 250 μmoles Tris–HCl buffer, pH 8.4, and the enzyme which had been dialysed against the appropriate buffer. After incubation at 37 °C for 10 min, the reaction was stopped and the amount of product was determined as described previously [2]. In the experiments where the enzyme was incubated with 125 μmoles of both L-serine and L-threonine in one assay, the amount of pyruvate and α-keto-butyrate formed was determined by the method of Flavin and Slaughter [4]. One enzyme unit is defined as the amount of enzyme which forms 1 μmole of pyruvate under the conditions mentioned. At low enzyme or PLP concentrations controls were performed with the direct assay, to exclude inactivation of the enzyme by L-serine [5].

Titration of the enzyme with PLP

PLP was added stepwise to cuvettes of 1-cm light path containing the apo-enzyme in the buffer indicated with added cations. Together with PLP, an equal amount of a 2-fold concentrated solution of apoenzyme was added to the enzyme cuvette to keep the enzyme concentration constant. A control cuvette containing buffer and cations received equal amounts of PLP and buffer. The increase of absorbance at 335 nm, where the Schiff's base between PLP and the apoenzyme has an absorption maximum [6], was used to determine the amount of PLP bound to the

enzyme. The measurements were performed in a Zeiss spectrophotometer PM Q2 with a thermostatted cell compartment.

Dissociation constants of Schiff's bases

Dissociation constants of the Schiff's bases of PLP with L-serine, L-threonine, and Tris ethanolamine were determined as described by Matsuo [7].

RESULTS

T/S ratio with both substrates together in the incubation

The T/S ratios reported in the literature [8–10] were determined with one substrate only, L-serine or L-threonine, in the incubation mixture. To obtain conditions which can be supposed to be more similar to those in the cytoplasm, we incubated the enzyme with both substrates together in the incubation mixture and measured the ratio of the two activities. In Table I the two kinds of T/S ratios are compared. The T/S ratio, obtained by incubating with L-serine or L-threonine separately, changes from 0.83 at a high enzyme concentration (10 μg) to 0.57 at a low enzyme concentration (1 μg). And, similarly, altering the PLP concentration in the incubation from 200 to 20 μM, decreases the T/S ratio from 0.82 to 0.49. On the other hand, if the enzyme is incubated with both substrates together, the change in the T/S ratio at different enzyme or PLP concentrations is no longer observed.

TABLE I

T/S ratio with L-threonine and L-serine separated or combined in the incubation at different PLP and enzyme concentrations. The activity was determined (a) with different enzyme concentrations (1 and 10 μg) at 200 μM PLP and (b) at different PLP concentrations (20 and 200 μM) with 3.8 μg of enzyme. In each case the enzyme was incubated with 0.1 M L-serine and with 0.1 M L-threonine separately and with 0.05 M L-serine and 0.05 M L-threonine combined. The amount of keto acids was measured as described in Materials and Methods.

incubation	T/S ratios			
	Enzyme concentration in the incubation (μg)		PLP concentration in the incubation (μM)	
	10	1	200	20
Substrates separated	0.86	0.57	0.82	0.49
Substrates combined	0.83	0.83	0.82	0.83

The fact that the T/S ratio is influenced by the PLP concentration suggested that the change of the T/S ratio might be explained by differences in the PLP binding constant of the enzyme under various conditions.

Influence of different cations on the PLP dissociation constant of the enzyme

Fig. 1 demonstrates the influence of monovalent cations on the PLP–enzyme dissociation constant obtained by measuring the activity at varying PLP concentrations. Whereas Na^+ seem to have little influence, K^+ and NH_4^+ greatly increase the binding of PLP to the enzyme. A similar study was presented in a previous paper [2],

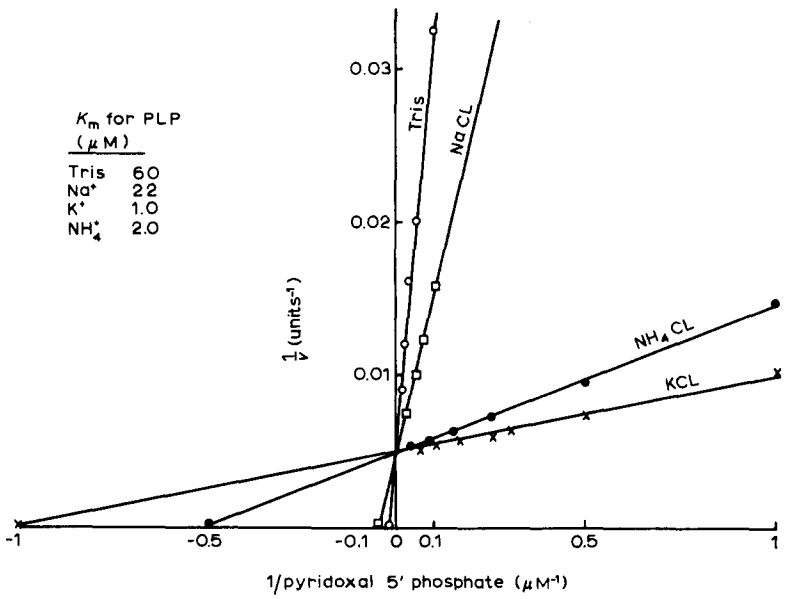

Fig. 1. Double reciprocal plots of L-serine dehydratase activity and PLP concentration in the presence of K^+, NH_4^+, Na^+ and Tris buffer. 12.5 μg (200 units) of apoenzyme were assayed in 0.1 M Tris buffer, pH 8.4, in the presence of 0.1 M NaCl, KCl or NH_4Cl with varying concentrations of PLP. The incubation was performed at 25 °C with L-serine as substrate.

but, because of a lower enzyme concentration used in those former experiments, some of the dissociation constants were considerably different (see Discussion). Pestaña [11] obtained similar results with a crude enzyme.

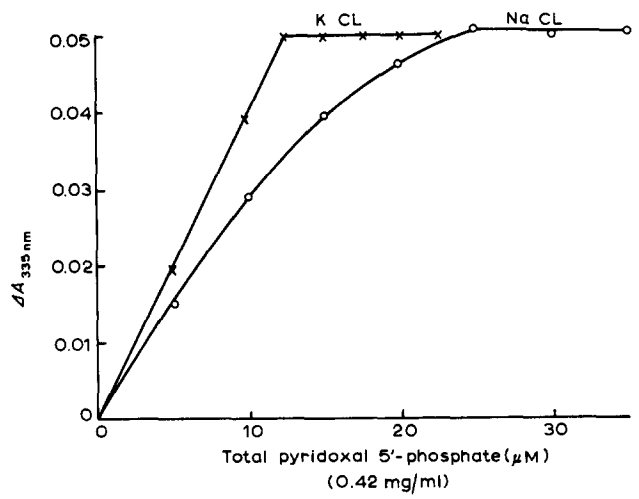

Fig. 2. Optical titration of apo-L-serine dehydratase with PLP in the presence of K^+ and Na^+. 0.45 mg/ml of apoenzyme, that is a 6.6 μM concentration of enzyme at a molecular wt of 68 000, were titrated with PLP at 335 nm at 25 °C in 0.1 M sodium pyrophosphate buffer, pH 8.4, and in the same buffer + 0.1 M KCl (○—○) as described under Materials and Methods.

We tried to confirm these results in the absence of the substrate by a more direct method.

As can be seen in Fig. 2, optical titration of the apoenzyme with PLP in sodium pyrophosphate buffer in the presence of 0.1 M KCl gives a straight line. The end point of the titration for a 6.6 μM enzyme solution is reached at 12.5 μM PLP, corresponding to 1.9 moles of coenzyme bound per mole of enzyme. This value agrees with the results of Nakagawa and Kimura [6] and Inoue et al. [12] obtained by equilibrium dialysis. 12.5 μM enzyme-bound PLP give an increase in absorbance of 0.05 at 335 nm. From this, a molar extinction coefficient of 4000 is calculated for enzyme-bound PLP. The values from the titration curve of the enzyme in sodium pyrophosphate buffer (Fig. 2) were used to determine a PLP–enzyme dissociation constant of approx. $2 \cdot 10^{-6}$ M with the double reciprocal plot of free and enzyme-bound PLP.

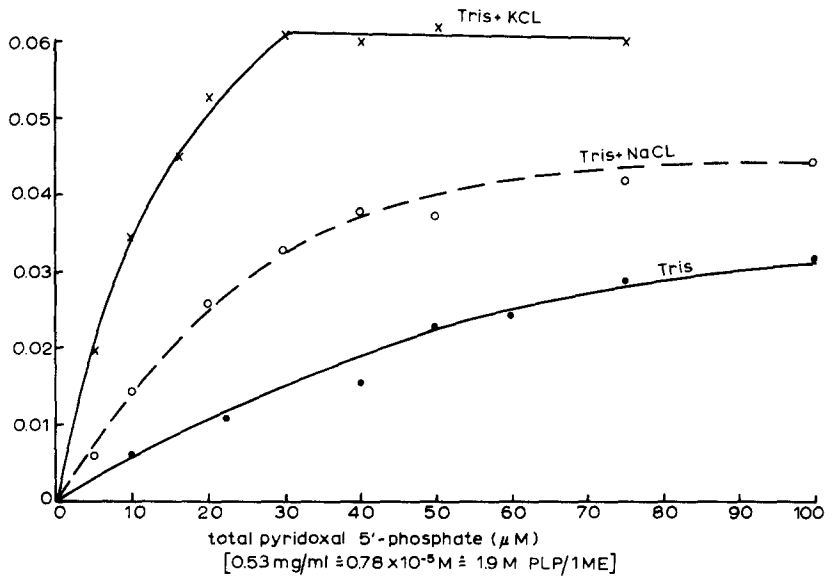

Fig. 3. Influence of K^+, Na^+, Tris buffer on the binding of PLP to the enzyme (optical titration). 0.53 mg/ml of apoenzyme were titrated optically with PLP at 335 nm at 25 °C in 0.1 M Tris buffer, pH 8.4, (●—●), in Tris buffer + 0.1 M KCl (×—×) and in Tris buffer + 0.1 M NaCl (○---○). For details of the titration see Materials and Methods.

Figs 3 and 4 show the titration curves and the double reciprocal plots, respectively, of the enzyme in Tris buffer, in Tris buffer + 0.1 M NaCl, and in Tris buffer + 0.1 M KCl. The dissociation constant in Tris buffer ($K_{Tris} = 100\,\mu M$) is considerably higher than that in sodium pyrophosphate buffer ($K_{Pyr} = 2 \cdot 10^{-6}\,\mu M$) (Fig. 2), indicating an inhibition of PLP binding by Tris buffer. Na^+ ($K_{Na} = 42\,\mu M$) has only a small influence on the dissociation constant, whereas K^+ ($K_K = 1.9\,\mu M$) greatly increases the binding of PLP to the enzyme.

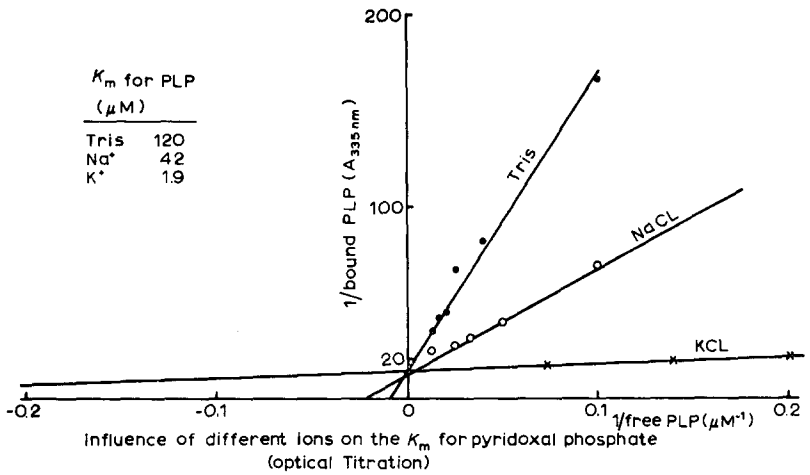

Fig. 4. Double reciprocal plots of enzyme-bound and free PLP in the presence of K^+, Na^+ and Tris buffer. The enzyme-bound PLP was calculated from the values in Fig. 4 with molar extinction coefficient for enzyme-bound PLP of 4000. Free PLP was obtained by subtraction of enzyme-bound PLP from total PLP. For experimental conditions see Fig. 3.

Influence of the enzyme concentration on the K_m for PLP

Another factor which influences the PLP dissociation constant is the enzyme concentration. Fig. 5 demonstrates that the dissociation constant at low enzyme concentration is six times larger than at high enzyme concentration. Moreover, V is about three times lower at the low enzyme concentration.

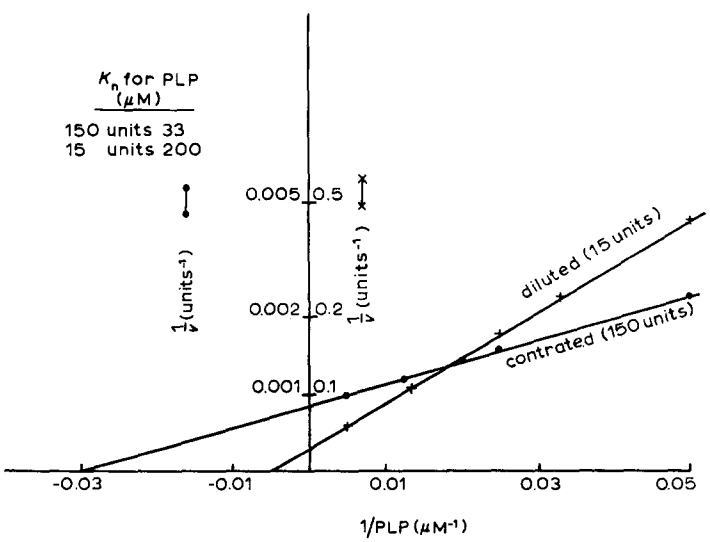

Fig. 5. Influence of the enzyme concentration on the K_m for PLP. 9.4 μg (150 units) and 0.94 μg (15 units), respectively, of apoenzyme were assayed in 0.1 M sodium pyrophosphate buffer, pH 8.4, at varying concentrations of PLP with L-serine as substrate. The K_m values for PLP calculated from the double reciprocal plots are: 9.4 μg of enzyme (●—●), 33 μM; 0.94 μg of enzyme (×—×), 200 μM.

Influence of substrates and Tris buffer on the K_m for PLP

The variation of the PLP dissociation constant with salts and enzyme concentration suggested that the enzyme was not in each case saturated with PLP under the assay conditions used. Therefore, a different effect of threonine and serine on the PLP binding to the enzyme could explain the change in the T/S activity ratio. In Table II the influence of different concentrations of both substrates on the dissociation constant was studied. With 10 mM L-serine or L-threonine the dissociation constants are lower than those with 50 and 100 mM of either substrate. Furthermore, the dissociation constants obtained with L-serine are lower than those measured with the corresponding concentration of L-threonine. We can conclude that both substrates, but L-threonine more markedly than L-serine, inhibit the binding of PLP to the enzyme.

TABLE II

Influence of different substrates (L-serine or L-threonine) and substrate concentrations on the K_m for PLP. 4.7 μg of apoenzyme were assayed in sodium pyrophosphate buffer with varying concentrations. The K_m values were calculated from double reciprocal plots of enzyme activity and PLP concentration.

Substrate conc. (mM)	K_m (μM) for PLP with	
	L-Serine	L-Threonine
10	7	17
50	21	62
100	62	103

Amino acids and Tris are known to form Schiff's bases with PLP, and thus they compete with the enzyme for its coenzyme. To be able to evaluate whether Schiff's base formation could be quantitatively responsible for the change of the apparent enzyme–PLP dissociation constant with different substrates, and to calculate the real dissociation constants, we determined the dissociation constants of the Schiff's bases with Tris, L-threonine, and L-serine under assay conditions (Table III). It can be seen that L-threonine, and to a similar degree $Tris^+$, binds PLP more than two times stronger than L-serine does.

TABLE III

Dissociation constants of the Schiff's bases of PLP with L-serine, L-threonine and Tris. The PLP dissociation constants were determined for L-serine and L-threonine in water brought to pH 8.4 with HCl, and for Tris in 0.1 M sodium pyrophosphate buffer, pH 8.4. The method of Matsuo [7] was used for the determination of the constants.

PLP dissociation constants (mM)		
Tris	L-Threonine	L-Serine
1.5	1.1	2.4

Calculation of the real enzyme–PLP dissociation constants

The PLP dissociation constants of the enzyme, measured by optical titration

in Tris buffer, are only apparent dissociation constants because they were determined in the presence of the competitive inhibitor of PLP binding: $Tris^+$. The real dissociation constants in the presence of K^+ or NH_4^+ cannot be measured directly by optical titration because they are too low, and titration would give a straight line. The real dissociation constants K can be calculated from the apparent dissociation constants K_{app} with the formula: $K_{app} = K\ (1 + [T]/K_T)$. The formula is obtained in the following way. Abbreviations: K_{app}, apparent PLP dissociation constant of the enzyme in the presence of $Tris^+$; K, real PLP–enzyme dissociation constant; K_T, $Tris^+$–PLP dissociation constant; [T], molar concentration of free $Tris^+$, approximately equal to the total Tris in this case; [E], molar concentration of free enzyme; [EP], molar concentration of enzyme–PLP complex; $[E]_t$, total molar concentration of enzyme; [P], molar concentration of free PLP; $[P]_t$, total molar concentration of PLP; [TP], molar concentration of $Tris^+$–PLP complex.

Beginning with five basic equations

$$\frac{[E]\cdot[P]}{[EP]} = K \tag{1}$$

$$\frac{[T]\cdot[P]}{[TP]} = K_T \tag{2}$$

$$\frac{[E]\cdot([P]_t - [EP])}{[EP]} = \frac{[E]\cdot[P]_t}{[EP]} - [E] = K_{app} \tag{3}$$

$$[P]_t = [EP] + [TP] + [P] \tag{4}$$

$$[E]_t = [EP] + [E] \tag{5}$$

it can be deduced that

$$[E] = \frac{[E]_t}{1 + \dfrac{[P]}{K}} \tag{6}$$

$$[EP] = [E]_t\cdot\left(1 - \frac{1}{1 + \dfrac{[P]}{K}}\right) \tag{7}$$

$$[P] = \frac{[P]_t}{1 + \dfrac{[T]}{K_T} + \dfrac{[E]}{K}} \tag{8}$$

By substitution of [E] and [EP] in Eqn 3 by Eqns 6 and 7 we obtain

$$K_{app} = \frac{[E]_t\cdot[P]_t}{\left(1 + \dfrac{[P]}{K}\right)\cdot[E]_t\left(1 - \dfrac{1}{1 + \dfrac{[P]}{K}}\right)} - [E] = \frac{[P]_t\cdot K}{[P]} - [E] \tag{9}$$

Substitution of [P] by Eqn 8 results in

$$K_{app} = \frac{[P]_t \cdot K\left(1 + \frac{[T]}{K_T} + \frac{[E]}{K}\right)}{[P]_t} - [E] = K\left(1 + \frac{[T]}{K_T}\right) \quad (10)$$

The real dissociation constants calculated in this way are: $1.5 \cdot 10^{-6}$ M in 0.1 M buffer, pH 8.4, $6 \cdot 10^{-7}$ M in buffer with 0.1 M NaCl in addition, $3 \cdot 10^{-8}$ M in buffer with 0.1 M KCl.

DISCUSSION

The constancy or variability of the ratio of the L-threonine and L-serine activities (T/S ratio) of animal L-serine dehydratases have been used as a criterion of whether or not both amino acids are degraded by a single enzyme [8–10]. Although it was shown that L-serine dehydratase from rat liver degrades both substrates, the T/S ratios reported for this enzyme differ from one author to the other [10]. In an earlier report we demonstrated a considerable variation of the T/S ratio with rat liver serine dehydratase [2]. To explain this change of the T/S ratio, it was necessary to measure the influence of various parameters on the PLP–enzyme dissociation constants.

The PLP–enzyme dissociation constant changes with the ionic environment. By kinetic determination, as well as by optical titration, we could show that K^+ and NH_4^+ increase the binding of PLP to the enzyme considerably, thus confirming earlier kinetical measurements [2, 11]. As $Tris^+$, L-serine, and L-threonine compete with the enzyme for PLP by forming Schiff's bases, constants determined in the presence of these agents are only apparent dissociation constants. This is also true for dissociation constants of serine dehydratase reported by Nakagawa and Kimura [6] and Pestaña [11]. The apparent dissociation constants which we measured by activity differ slightly from the optically measured constants. The reason is that the former ones were determined in the presence of two inhibitors, Tris and L-serine, whereas with the determination of the latter Tris was the only inhibitor. But calculation of the real dissociation constants from both kinds of apparent dissociation constants lead to identical results.

By optical titration we could measure the real PLP–enzyme dissociation constant in 0.1 M sodium pyrophosphate buffer to be $K_P = 2 \cdot 10^{-6}$ M. This value is in good agreement with the real dissociation constant for 0.1 M buffer $K = 2 \cdot 10^{-6}$ M which was calculated from the apparent dissociation constant in the presence of $Tris^+$. The real dissociation constant in the presence of K^+ or NH_4^+ cannot be measured directly by optical titration, but can easily be calculated from the apparent dissociation constants measured in the presence of $Tris^+$. In our opinion, this method could be useful with other PLP–enzymes, too.

The PLP–enzyme dissociation constant is not only influenced by K^+ or NH_4^+, but also by the enzyme concentration. At low enzyme concentration, the dissociation constant is about ten times higher than at high enzyme concentration. The reason could be that the enzyme dissociates into two subunits upon dilution in the absence of K^+ or NH_4^+ as shown previously by Sephadex gel filtration and sucrose

gradient centrifugation [3], and that the subunits being of a certain activity have a higher dissociation constant than the native enzyme.

Partially similar effects of monovalent cations have been shown for tryptophanase by Morino and Snell [13]. As with L-serine dehydratase, K^+ increases the PLP binding constants for tryptophanase. But in contrast to its action on L-serine dehydratase, K^+ promotes the dissociation of tryptophanase into its subunits at 5 °C.

The apparent enzyme–PLP dissociation constants are further influenced by the substrates. When the constants are measured by the activity on L-threonine at different PLP concentrations, constants about three times higher are obtained than by measuring the activity on L-serine. The reason is that the affinity of L-threonine to form a Schiff's base with PLP is more than two times greater than that of L-serine.

These facts taken together can explain the change of the T/S ratio. When PLP is in excess, the enzyme will be saturated with PLP in the presence of L-threonine, as well as in the presence of L-serine, and the T/S ratio will only depend on the K_m and the V for both substrates. But when the PLP concentration is not about ten times in excess of the apparent dissociation constant of the enzyme, there will, because of the different strong competition of L-threonine and L-serine for PLP, result a smaller amount of active holoenzyme with L-threonine than with L-serine in the test. The T/S ratio will decrease. This is the case at low PLP concentrations, at low enzyme concentrations (because of a higher dissociation constant of the subunits), and in the presence of Tris. If, on the other hand, the enzyme is incubated with both substrates together, there will be the same amount of enzyme acting on L-serine as on L-threonine. The T/S ratio will be constant. These results argue against a physiological significance of the change in the T/S ratio, if one does not assume that separated pools of threonine and serine exist in the cell.

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