THE BINDING OF THIAMINE PYROPHOSPHATE WITH TRANSKETOLASE IN EQUILIBRIUM CONDITIONS

G.A. Kochetov, N.K. Tikhomirova, P.P. Philippov

Laboratory of Bioorganic Chemistry, Lomonosov State University, Moscow 117234, USSR

Received February 10, 1975

SUMMARY:

The binding between thiamine pyrophosphate and transketolase, purified from baker's yeast, in equilibrium conditions has been studied. In the presence of Ca^{2+}, the enzyme molecule has been shown to possess two binding sites for the coenzyme, whose dissociation constants are 3.2×10^{-8} and 2.5×10^{-7}M; besides, there are site(s) where the binding of the coenzyme is less firm. In the presence of Mg^{2+}, a positive cooperative interaction between the binding sites of thiamine pyrophosphate has been observed. Regardless of the cation used, the major part of the catalytic activity of the transketolase molecule manifests itself in the binding of one molecule of the coenzyme.

In 1961 it was shown by Racker and Datta (1) that a transketolase (TK) molecule may bind from 2 to 9 molecules of thiamine pyrophosphate (TPP). Later on there were some works in which the interaction of TK with TPP and its analogues were studied (2-10), but it was only in two works (6,9) that the number of TPP binding sites in TK was determined, and it was equal to 2. In both cases the enzyme was preincubated in the presence of TPP and Mg^{2+}, the free cofactors were then removed by gel filtration on Sephadex, after which the quantity of protein-bound TPP was determined. In the present paper the binding of TPP with TK purified from baker's yeast was studied in the presence of Ca^{2+} and Mg^{2+} under the equilibrium conditions, i.e. the quantity of protein-bound TPP was determined in the presence

of an equilibrium concentration of the coenzyme.

METHODS

TK, after having been purified by the method of Racker et al. (11), was fractionated twice or thrice with a saturated solution of ammonium sulphate (pH 7.6); the resulting preparations of the enzyme were homogenous, as proved by the data of polyacrylamide gel electrophoresis. The specific activity of the preparations was 10 U if measured directly, or 20 U if prior to activity measurements TK was preincubated with TPP and metal ion (temperature 25°C in both cases). The quantity of protein was determined by the optical density value of TK solutions at 280 nm (the optical density value of 1% TK solution at 280 nm is 14.5 (12)) or by the method of Lowry (13). In the latter case bovine serium albumin was used as standard protein and the results of the measurements were multiplied by a factor of 0.7. The TK activity (14) and the quantity of TPP (1) were determined as described previously. The binding of TPP with TK was studied as described in (15) with the help of Sephadex G-50 columns (1.4 x 28 cm), which were equilibrated with 2.5 mM potassium-phosphate buffer (pH 7.6), containing 1.0 mM $CaCl_2$ or $MgCl_2$ and the required concentrations of TPP (temperature 25°C). In the presence of Ca^{2+}, the TPP concentration was varied from 0.02 - 3.6 μM, in the presence of Mg^{2+}- from 0.2 to 3.6 μM; the duration of gel filtration was 2.5 hours in the former case and 3.5 hours in the latter.

RESULTS

The results on TPP-TK binding were treated in r/A vs r coordinates (16), where r is the value of the ratio of the number of protein-bound TPP to the number of moles of TK (molecular

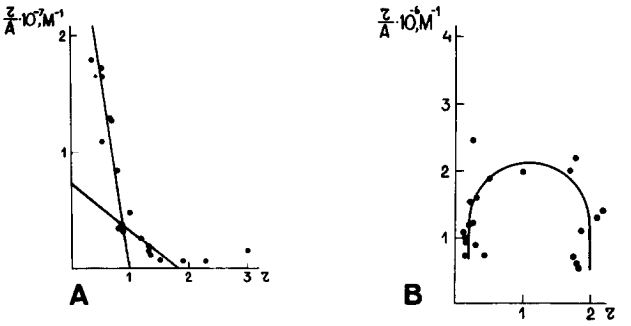

Fig. 1. Binding of TPP with TK in the presence of Ca^{2+} (A) and Mg^{2+} (B). For experimental conditions see "Methods". Straight lines in Fig. 1A are drawn by the least squares method.

---

weight 140,000 (1)), A is the concentration of free TPP (in our case the concentration of free coenzyme is practically equal to its total concentration). The results obtained in the presence of Ca^{2+} (Fig. 1A) should be interpreted as indicating that in the TK molecule there are several independent sites possessing a different affinity to TPP. The site where the coenzyme is bound most firmly has a dissociation constant $K' = 3.2 \times 10^{-8}$ M, the site of less firm binding - $K'' = 2.5 \times 10^{-7}$ M; in addition, there is a site(s) whose dissociation constant is unknown, but its value is undoubtedly much higher than in the first two cases. It is not excluded, however, that the dependence shown in Fig. 1A is indicative of negative cooperative interaction between the TPP binding sites (17).

The character of the binding between TPP and TK in the presence of Mg^{2+} (Fig. 1B) is basically different from that in the case of Ca^{2+}. The bell-shaped curve of the r/A vs r dependence reflects the positive cooperative interaction between the coenzyme binding sites (17). An approximate extrapolation of the experimental curves to the abscissa gives an r values close to 2. In this case, unlike the experiments with Ca^{2+}, no direct deter-

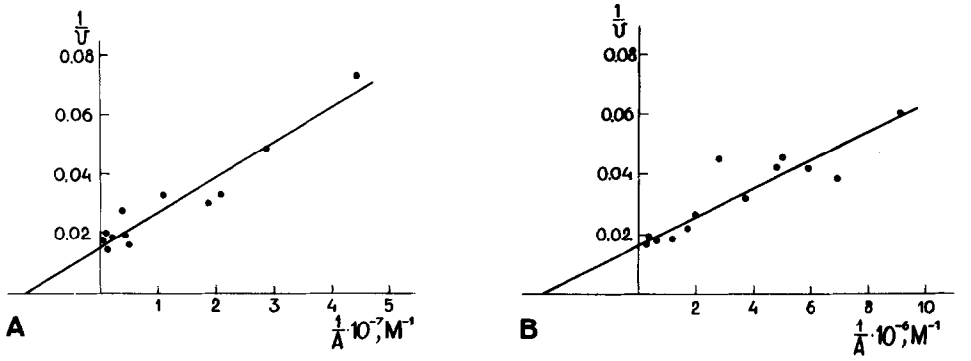

Fig. 2. Dependence of TK activity on TPP concentration in the presence of Ca^{2+} (A) and Mg^{2+} (B). TK activity was determined after gel filtration in Sephadex G-50 equilibrated by a buffer containing TPP and the cation (for details see "Methods"). Straight lines are drawn by the least squares method.

---

mination of the K_D value is possible, but this can be done by studying the dependence of transketolase activity (v) on TPP concentration (Fig. 2B). The dissociation constant (K_D^a) deter-

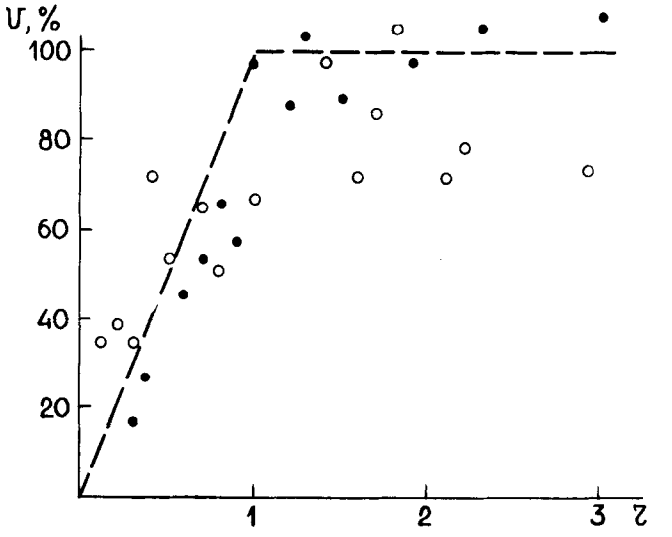

Fig. 3. Dependence of TK activity on the amount of protein-bound TPP. Reconstruction of holo-TK was carried out in the presence of Ca^{2+}(●) or Mg^{2+} (o); (- - -) theoretical graph for the case when the enzyme molecule contains only one active center and value K_D for active TPP molecule is much lower than for nonactive one(s). Activity measured in the presence of the optimal concentrations of the cation and TPP was taken for 100%.

mined in this way equals 3.0×10^{-7} M; a similar calculation in the case of Ca^{2+} (Fig. 2A) gives for TPP a value of K_D^a equal to 7.4×10^{-8}M.

In the experiments in which the binding of TPP with the apoenzyme was studied, we also measured the TK activity at every given value of r . It is seen in Fig. 3 that in the presence of Ca^{2+}, the major part, if not all, of the catalytic activity of the TK molecule is revealed in the binding of one TPP molecule; in the binding of the other molecule of the coenzyme, the TK activity increases insignificantly. Although the results obtained in the presence of Mg^{2+} are not so unambiguous, it is nevertheless evident that here too the major part of the activity should be referred to the first of the two bound TPP molecules.

DISCUSSION

It has been demonstrated that the maximum catalytic activity of TK reveals itself when approximately 2 moles of TPP become bound to a mole of the enzyme (6,9). If no metal is added on reconstruction and the quantity of bound TPP is measured after the free coenzyme has been removed, the enzyme activity increases linearly as r changes from 0 to 2. In the presence of Mg^{2+}, the linearity is not observed so strictly, and at r=1 over 60% of the maximal TK activity was revealed (6). However, if the removal of free TPP is carried out on larger columns and for a longer time, the percentage of the TK activity at r=1 is at least 70% regardless of whether the metal (calcium and/or magnesium) is added or not (our unpublished data). These data plus the data of this study prompts one to the following conclusions: 1) either in the TK molecule there is only one active site and the other TPP binding site(s) performs a different

function; 2) or else in the TK molecule there are two active sistes possessing different catalytic efficiencies.

Apart from the relationship between the TK activity and the amount of TPP bound to the enzyme, attention should be paid to the different character of TPP-TK binding in the presence of Ca^{2+} and Mg^{2+} (Fig. 1A and 1B). In the case of Mg^{2+} one may speak with certainty about the positive cooperativity between the TPP binding sites, whereas with Ca^{2+} there is either a negative cooperative interaction between the TPP binding sites, or in the TK molecule there are several independent TPP binding sites possessing different affinities to the coenzyme. (The case when an enzyme may exists in several, possibly interconverting, forms with different affinity to the ligand to be bound, is not discussed here). If the TPP binding sites are mutually independent, the values of dissociation constants may be calculated. The K_D' and K_D'' values thus obtained for TPP in the presence of Ca^{2+} are equal to 3.2×10^{-8} M and 2.5×10^{-7} M, respectively. The K_D^a value in the presence of Ca^{2+} determined from the 1/v vs 1/A dependence is 7.4×10^{-8} M and should be referred to the first, "stronger", binding site if the hypothesis of one active centre is valid, i.e. the K_D' and K_D^a values should coincide. If the suggestion about two active sites of different effectivity is true, than, strictly speaking, the 1/v vs 1/A curve should be nonlinear (i.e. $K_D' \neq K_D''$). But if this nonlinearity is weakly expressed and not taken into account when drawing the graph, then unequality $K_D' < K_D^a < K_D''$ will be valid, which is in fact the case. This is to a certain extent an argument in favour of the second hypothesis. No such comparison is possible for the experiments with Mg^{2+}, as only $K_D^a = 3.0 \times 10^{-7}$ M is known. But, remembering that in the experiments

with Ca^{2+} the values of K_D^i and K_D^a differ two-fold at the most, the K_D^i value for TPP in the presence of Mg^{2+} may be estimated from the corresponding K_D^a.

ACKNOWLEDGMENTS

The authors thanks T.Kheifets for assistance in preparation of this paper.

REFERENCES

1. Datta, A.G. and Racker, E. (1961) J.Biol.Chem. 236, 624-628.
2. Kochetov, G.A., Usmanov, R.A. and Merzlov, V.P. (1970) FEBS Letters 9, 265-266.
3. Kochetov, G.A. and Usmanov, R.A. (1970) Biochem.Biophys.Res. Commun. 41, 1134-1140.
4. Kochetov, G.A., Izotova, A.E. and Meshalkina, L.E. (1971) Biochem.Biophys.Res.Commun. 43, 1198-1203.
5. Kochetov, G.A. and Izotova, A.E. (1972) Dokl.AN USSR 205, 986-988.
6. Kochetov, G.A., Izotova, A.E., Philippov, P.P. and Tikhomirova, N.K. (1972) Biochem.Biophys.Res.Commun. 46, 616-620.
7. Heinrich, C.P., Steffen, H., Janser, P. and Wiss, O. (1972) Eur.J.Biochem. 30, 533-541.
8. Kochetov, G.A. and Izotova, A.E. (1973) Biokhimiya 38, 552-559.
9. Heinrich, C.P. (1973) Experientia 15, 1227-1228.
10. Heinrich, C.P., Schmidt, D. and Noack, K. (1974) Eur.J.Biochem. 41, 555-561.
11. Srere, P.A., Cooper, J.R., Tabachnick, M. and Racker, E. (1958) Arch.Biochem.Biophys. 74, 295-305.
12. Heinrich, C.P., Noack, K. and Wiss, O. (1972) Biochem.Biophys. Res.Commun. 49, 1427-1432.
13. Lowry, O.H., Rosenrough, N.J., Farr, A.L. and Randall, R.J. (1951) J.Biol.Chem. 193, 265-275.
14. Racker, E. (1961) in: The Enzymes, vol. 5, pp. 397-406, Academic Press, New York and London.
15. Hummel, J.P. and Dreyer, W.J. (1962) Biochim.Biophys.Acta 63, 530-532.
16. Scatchard, G. (1949) Ann.N.Y.Acad.Sci. 51, 660-672.
17. Cook, R.A. and Koshland, D.E. (1970) Biochemistry 9, 3337-3342.