Biochimica et Biophysica Acta, 527 (1978) 249—255

BBA 68562

THE AFFINITY CHROMATOGRAPHY OF TRANSKETOLASE

TERRY WOOD and SUSAN FLETCHER

Department of Biochemistry, University of Rhodesia, P.O. Box MP 167, Mount Pleasant, Salisbury (Rhodesia)

(Received April 14th, 1978)

Summary

A number of possible affinity adsorbents for transketolase (sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphateglycolaldehydetransferase, EC 2.2.1.1) were prepared. The behaviour of the enzyme from *Candida utilis* and from Baker's yeast on columns of these and of Blue Sepharose CL-6B was examined, together with the behaviour of the contaminating enzyme, ribulose 5-phosphate 3-epimerase (EC 5.1.3.1). A procedure for removing bound thiamine pyrophosphate by dialysis against EDTA was developed. The competitive inhibition of transketolase by oxythiamine and neopyrithiamine was measured and the K_i values obtained of 1.4 and 4.3 mM, respectively, were compared with the affinity of adsorbents prepared from these two inhibitors.

Adsorbents containing bound thiamine pyrophosphate were relatively ineffective but those containing epoxy-linked neopyrithiamine and D-ribose 5-phosphate adsorbed the enzyme at pH 7.4 and it could be eluted in a specific manner.

Introduction

During the course of an investigation into methods of purifying transketolase (EC 2.2.1.1) from *Candida utilis* and removing contaminating ribulose 5-phosphate 3-epimerase (EC 5.1.3.1) suitable affinity adsorbents were sought. The thiamine pyrophosphate-agarose described by Matsuura et al. [1] was unstable and had no affinity for transketolase, so other adsorbents were prepared by coupling suitable compounds to epoxy-activated Sepharose [2] and to diazotised *p*-aminobenzamidohexyl-Sepharose [3] and their properties investigated.

Materials

Sepharose derivatives were supplied by Pharmacia, Uppsala, Sweden. *C. utilis* and other reagents were from Sigma, U.S.A. Baker's yeast was obtained locally.

Methods

Preparation of epoxy-coupled Sepharose 6B derivatives

3 ml fresh epoxy-activated Sepharose were suspended in 2 ml 70 mM borate buffer/120 μmol derivative and shaken in a constant temperature bath. Pyrithiamine and thiamine pyrophosphate were coupled at pH 9.0 and 40° C; D-ribose 5-phosphate was coupled at pH 8.5 and 33° C. After 65 h, the gel was filtered, washed and excess groups blocked with ethanolamine as described by the manufacturers [2].

Preparation of diazo-coupled Sepharose 4B derivatives

Aminohexyl-Sepharose 4B was converted to *p*-aminobenzamidohexyl-Sepharose 4B by treatment with *p*-nitrobenzoyl azide [4] and reduction with sodium dithionite [5]. 3-ml portions of the gel were diazotised and coupled to thiamine pyrophosphate, thiamine, oxythiamine, and neo-pyrithiamine as described by O'Brien et al. [3].

Analyses of affinity adsorbents

Ribose 5-phosphate-Sepharose (0.5 ml) was digested with 0.5 ml 2 M HCl for 10 min at 100° C to dissolve the matrix. The solution was brought to pH 8.5 with NaOH, mixed with 1 ml 100 mM triethanolamine-chloride buffer (pH 8.5) and $MgCl_2$ added to 5 mM. 0.5 unit alkaline phosphatase was added and the mixture incubated 6 h at 37° C. An aliquot was withdrawn and the phosphate measured by the method of Fiske and SubbaRow [6]. Epoxy- and diazo-linked thiamine pyrophosphate-Sepharose were hydrolyzed directly with 1 M HCl at 100° C until, after 2 h, no more phosphorus was liberated.

Preparation of transketolase

The enzyme was prepared from *C. utilis* and the 30—40% acetone fraction was used [7]. Baker's yeast was autolysed [8] and the 33—50% acetone fraction prepared [9]. Transketolase and ribulose 5-phosphate 3-epimerase were assayed as before [7]. The coenzyme was removed by dialysis.

Studies of oxythiamine and pyrithiamine inhibition

Samples of apo-transketolase were incubated with the inhibitor at room temperature in the presence of all the assay constituents except the coenzyme. After 30 min, the coenzyme was added and initial velocities at 30° C were measured at 0.2 μM and 0.6 μM thiamine pyrophosphate. Concentrations of oxythiamine ranged from 0 to 2 mM and of neo-pyrithiamine from 0 to 10 mM.

Chromatography on affinity columns

Dialysed enzyme samples were run through short columns containing 3 ml adsorbent. In the case of adsorbents containing thiamine pyrophosphate, 2 mM $MgCl_2$ was added to the sample and buffers and 14 ml adsorbent were used. The columns were eluted with salts or with specific eluents and 4-ml fractions were collected and assayed for enzyme activities.

Two sets of conditions were tested: (1) room temperature and pH 7.4 in 10

mM triethanolamine-chloride buffer; (2) 10 mM imidazole buffer, 2 mM $MgCl_2$, 1 mM mercaptoethanol and 10% glycerol at pH 6.0 and 5°C [10]. In some experiments imidazole was replaced by acetate at pH 5.5 or pH 6.0.

Results

Efficiency of the coupling procedures

All the procedures were effective as deduced from the appearance of colour in the case of diazo-coupled adsorbents, from the test with 2 : 4 : 6 trinitrobenzene sulphonate [5], and the development of fluorescence with the thiochrome reagent [11] in the case of thiamine pyrophosphate-Sepharoses. Diazo-coupled thiamine pyrophosphate-Sepharose contained 1.8 μmol pyrophosphate groups per ml of gel 6 months after its preparation and the epoxycoupled thiamine pyrophosphate-Sepharose 6.3 μmol/ml after 2 months. The ribose 5-phosphate Sepharose contained 1 μmol phosphate groups per ml after 6 months.

Removal of the bound coenzyme

In contrast to an earlier report [7], thiamine pyrophosphate could only be removed from concentrated enzyme solutions by exhaustive dialysis against 10 mM EDTA/10 mM triethanolamine-chloride buffer (pH 7.6). If EDTA was omitted, samples assayed in the absence of added TPP retained up to 25% of their potential activity (assayed at 0.1 mM thiamine pyrophosphate). Up to 95% of the potential activity of the enzyme sample could be removed by dialysis against buffer alone, however, if the enzyme solution was dilute (Table I).

Inhibitor constants

Typical Dixon plots for competitive inhibition were obtained and K_i values of 1.4 mM for oxythiamine and 4.3 mM for pyrithiamine were measured.

Adsorption of transketolase to affinity adsorbents

The results with transketolase from *C. utilis* are summarized in Table II. When there was no adsorption, no greater affinity was shown by the enzyme from Baker's yeast.

TABLE I

THE REMOVAL OF THIAMINE PYROPHOSPHATE BY DIALYSIS AT pH 7.6

TPP, thiamine pyrophosphate.

Dialysis buffer	Protein concentration mg/ml	Enzyme concentration mg/ml *	% activity when assayed in absence of added TPP
10 mM triethanolamine chloride	6.4	0.38	6.5
10 mM triethanolamine chloride	0.6	0.03	1.0
10 mM EDTA in 10 mM triethanolamine chloride	8.5	0.37	1.0

* A specific activity of 15 units/mg was assumed for pure *C. utilis* transketolase as reported by Specht [10].

TABLE II

ADSORPTION OF TRANSKETOLASE FROM *C. UTILIS* BY VARIOUS ADSORBENTS

n.i. = not investigated; + = adsorption; TPP, thiamine pyrophosphate.

	Conditions	
	pH 6.0, 5°C	pH 7.4, 20—25°C
Epoxy-coupled		
TPP-Sepharose 6B	—	—
Ribose 5-phosphate-Sepharose 6B	+	+
Oxythiamine-Sepharose 6B	—	—
Neopyrithiamine-Sepharose 6B	+	+
Diazo-coupled		
TPP-Sepharose 4B	—	—
Thiamine-Sepharose 4B	—	—
Oxythiamine-Sepharose 4B	—	—
Neopyrithiamine-Sepharose 4B	—	—
Blue-Sepharose CL-6B	—	n.i.

Epoxy-coupled pyrithiamine-Sepharose 6B

Transketolase and ribulose 5-phosphate 3-epimerase were completely adsorbed. At pH 7.4, transketolase was not eluted by thiamine pyrophosphate alone, but was eluted when magnesium was added (Fig. 1).

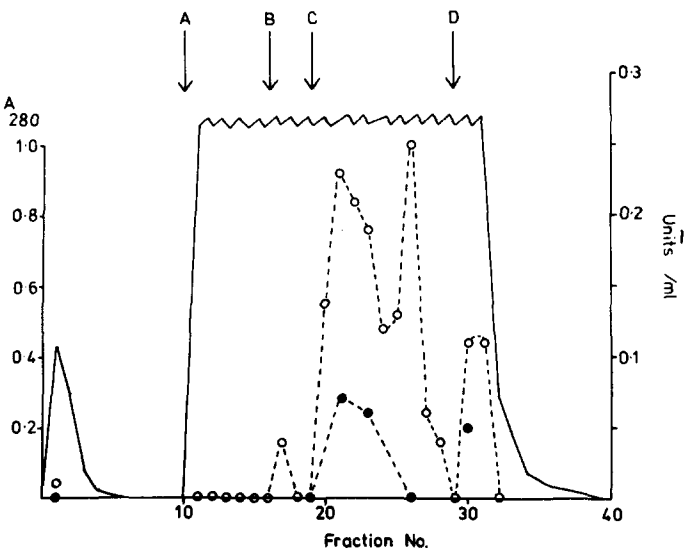

Fig. 1. Chromatography on epoxy-coupled pyrithiamine-Sepharose at room temperature and pH 7.4. The sample was applied at pH 7.4 and washed through with 10 mM triethanolamine/chloride buffer. Elution was carried out with 10 mM triethanolamine/chloride buffer (pH 7.4) to which the following additions had been made: A = 25 mM KCl + 4 mM TPP; B = 25 mM KCl + 4 mM TPP + 4 mM $MgCl_2$; C = 40 mM KCl + 4 mM TPP + 4 mM $MgCl_2$; D = 500 mM KCl; o- - - - -o, transketolase activity; •- - - - -•, ribulose 5-phosphate 3-epimerase activity; ———, absorbance at 280 nm in a 1-cm cell (A_{280}).

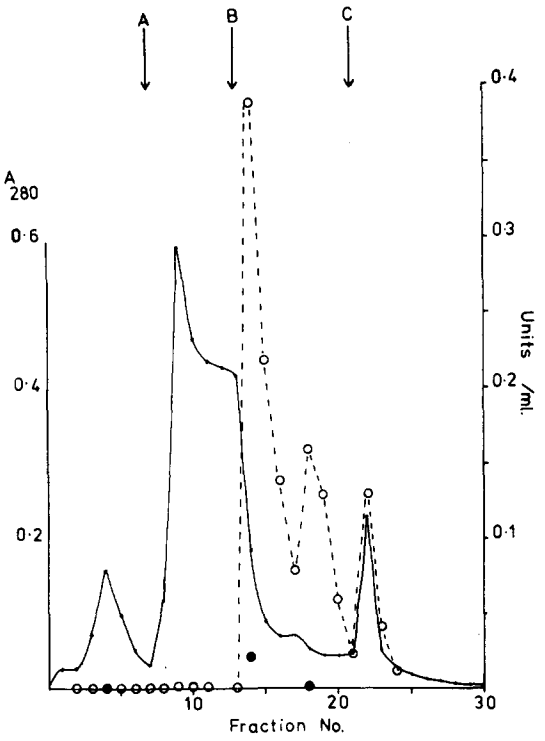

Fig. 2. Chromatography on epoxy-coupled ribose 5-phosphate-Sepharose at 5°C and pH 6.0. The sample was applied at pH 6.0 and washed through with 10 mM imidazole-chloride/2 mM $MgCl_2$/1 mM mercaptoethanol/10% glycerol (pH 6.0). Elution was carried out with 50 mM triethanolamine/chloride buffer (pH 7.4) to which the following additions had been made: A = 0.1 mM TPP + 2 mM $MgCl_2$ + 1 mM mercaptoethanol; B = 2 mM ribose 5-phosphate + 2 mM $MgCl_2$ + 1 mM mercaptoethanol; C = 500 mM KCl. o- - - - -o, transketolase activity; •- - - - -•, ribulose 5-phosphate 3-epimerase activity; ———, absorbance at 280 nm in a 1-cm cell (A_{280}).

Epoxy-coupled ribose 5-phosphate-Sepharose 6B

Both enzymes were completely adsorbed. The transketolase was not removed by 0.1 mM thiamine pyrophosphate/50 mM triethanolamine/chloride buffer (pH 7.4), but was specifically eluted by 2 mM ribose 5-phosphate/2 mM $MgCl_2$ in the same buffer (Fig. 2).

Column buffering

Although binding of transketolase to thiamine pyrophosphate-containing adsorbents was observed in some experiments at pH 6.0, in other experiments no binding could be obtained. This variability was eventually traced to an inadequate buffering of the adsorbent column with binding occurring when pH values of 5.5 or below were measured in samples of the column effluent. The conditions described by Specht [10] employ a cationic buffer (imidazole) to buffer an adsorbent containing anionic (pyrophosphate) groups. When imidazole starting buffer at pH 6.0 was passed through a column of diazo-coupled or epoxy-coupled thiamine pyrophosphate-Sepharose, which had been previously equilibrated with 100 mM imidazole chloride or 100 mM sodium

acetate buffer at pH 6.0, we found that the pH of the eluate first rose above 6.0 and then fell to a value of 5.0. This phenomenon caused us to observe a spurious binding of the enzyme at a nominal pH of 6.0 which did not occur when the anionic buffer acetate was used both to wash the column and to replace imidazole in the pH 6.0 starting buffer. However, as shown below, both types of TPP-Sepharose adsorbed transketolase from acetate buffer at pH 5.5.

Epoxy-coupled thiamine pyrophosphate-Sepharose

There was no significant adsorption of transketolase or of ribulose 5-phosphate 3-epimerase at pH 6.0 or above. However, in acetate buffer at pH 5.5 and 5°C some 75% of the transketolase was adsorbed whereas the epimerase passed through the column. Part of the adsorbed transketolase was eluted by 0.2 mM thiamine pyrophosphate (pH 5.5) and the remainder was eluted by triethanolamine buffer (pH 7.5). When 0.2 mM xylulose 5-phosphate and 0.2 mM ribose 5-phosphate were included in the enzyme placed on the column in acetate buffer at pH 6.0, some 30% of the transketolase remained bound to the column. The adsorbed transketolase and some non-enzyme protein were eluted at pH 7.5 by 50 mM triethanolamine buffer.

Diazo-coupled thiamine pyrophosphate-Sepharose

No significant adsorption occurred at pH 6.0 or above. With acetate starting buffer at pH 5.5 and 5°C, approx. 94% of the transketolase was adsorbed together with considerable amounts of epimerase. The adsorbed transketolase was not eluted by 0.2 mM thiamine pyrophosphate in starting buffer, but both enzymes were eluted by triethanolamine buffer at pH 7.5.

Discussion

If the enzyme solution were sufficiently dilute, thiamine pyrophosphate could be removed almost completely by dialysis against buffer alone. However, more concentrated solutions required dialysis against EDTA (Table I). Cavalieri et al. [12] have shown that the Baker's Yeast enzyme can dissociate into its constituent subunits at concentrations below 0.1 mg/ml of enzyme in the absence of thiamine pyrophosphate. Thus, it appears that at low enzyme concentrations the *C. utilis* enzyme dissociated sufficiently to allow the coenzyme to be removed by simple dialysis against buffer, whereas at higher enzyme concentrations the presence of EDTA was necessary. Magnesium is known to be involved in the binding of thiamine pyrophosphate to transketolase [13] and EDTA probably acts by chelating magnesium and allowing the thiamine pyrophosphate to be released.

The binding of thiamine pyrophosphate and magnesium to apotransketolase is much stronger below pH 6.4 [14] and both Specht [10] and Klein and Brand [15] claimed to have obtained affinity binding of transketolase to diazo-coupled thiamine pyrophosphate-Sepharoses at pH 6.0. Although some binding of transketolase to thiamine pyrophosphate-containing matrices was observed at this pH, it was traced to inadequate buffering of the column. With correctly buffered columns no adsorption took place until the pH was lowered to 5.5, a pH at which the enzyme will bind to cation exchangers such as cellulose phos-

phate (Wood, T., unpublished data) and carboxymethyl-Sephadex [16]. In the case of epoxy-coupled thiamine pyrophosphate-Sepharose affinity binding may have played a role, judged by elution of the enzyme with thiamine pyrophosphate at pH 5.5, but this did not occur with the diazo-coupled thiamine pyrophosphate-Sepharose.

Oxythiamine pyrophosphate binds more strongly to apotransketolase than pyrithiamine pyrophosphate [14] and the inhibitor constants presented here confirm a similar order for the free bases. It is likely that the coupling to the epoxy-activated Sepharose took place with the aliphatic primary hydroxyl group of oxythiamine rather than with the ring hydroxyl. Consequently, oxythiamine would be attached to the Sepharose 'the wrong way round' and so produce an ineffectual adsorbent.

The strong binding of transketolase and proteins in general to epoxy-coupled pyrithiamine-Sepharose may be due to the strong charge transfer interaction of pyrithiamine with protein tryptophan residues [17]. Transketolase could be eluted specifically, however by the addition of Mg-thiamine pyrophosphate to a concentration of salt that would not elute the enzyme in its absence.

Affinity adsorbents containing thiamine pyrophosphate have proved disappointing, probably due to the weak binding of thiamine pyrophosphate to the enzyme. The strength of binding could be increased in the presence of substrates and by lowering the pH to 5.5. At the latter pH, however, the enzyme is unstable and binding by cation exchange occurs. The adsorption of the enzyme by epoxy-coupled ribose 5-phosphate-Sepharose illustrates the strong affinity of the substrate site for ribose 5-phosphate and this adsorbent was very effective at pH 7.4 where the enzyme is completely stable.

Acknowledgement

We are indebted to Dr. J.A.A. Gooch for the preparation of *p*-nitrobenzoyl azide.

References

1 Matsuura, A., Iwashima, A. and Nase, Y. (1974) Methods Enzymol. 34B, 303—304
2 Pharmacia Fine Chemicals Publication (1976) Affinity Chromatography, Principles and Methods, Pharmacia Fine Chemicals, Uppsala
3 O'Brien, T.A., Schrock, H.L., Russell, P., Blake, R. and Gennis, R.B. (1976) Biochim. Biophys. Acta 452, 13—29
4 Munch-Petersen, J. (1963) Org. Synth. IV, 715—717
5 Cuatrecasas, P. (1970) J. Biol. Chem. 245, 3059—3065
6 Fiske, C.M. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375—400
7 Kiely, M.E., Tan, E.L. and Wood, T. (1969) Can. J. Biochem. 47, 455—460
8 Srere, P., Cooper, J.R., Tabachnik, M. and Racker, E. (1958) Arch. Biochem. Biophys. 74, 295—305
9 Haba, G. de la and Racker, E. (1955) Methods Enzymol. 1, 375—380
10 Specht, W. (1977) Doctoral Dissertation, Georg-August-Universitat zu Göttingen
11 Hawk, P.B., Oser, B.L. and Summerson, W.H. (1954) Practical Physiological Chemistry, 13th edn., p. 1151, J. and A. Churchill, London
12 Cavalieri, S.W., Neet, K.E. and Sable, H.Z. (1975) Arch. Biochem. Biophys. 171, 527—532
13 Datta, A.G. and Racker, E. (1961) J. Biol. Chem. 236, 617—623
14 Heinrich, P.C., Steffen, H., Janser, P. and Wiss, O. (1972) Eur. J. Biochem. 30, 533—541
15 Klein, H. and Brand, K. (1977) Hoppe-Seyler's Z. Physiol. Chem. 358, 1325—1337
16 Kochetov, G.A. and Chernov, N.N. (1971) Biokhimiya 36, 1168—1174
17 Heinrich, C.P., Schmidt, D. and Noack, K. (1974) Eur. J. Biochem. 41 555—561