PHOSPHOTRANSACETYLASE ASSOCIATED WITH THE PYRUVATE DEHYDROGENASE COMPLEX FROM THE NITROGEN FIXING *AZOTOBACTER VINELANDII*

T.W. BRESTERS, J. KRUL, P.C. SCHEEPENS and C. VEEGER
Department of Biochemistry, Agricultural University, Wageningen, The Netherlands

Received 6 March 1972

1. Introduction

During our efforts to investigate how reducing equivalents and energy are supplied, the involvement of pyridine nucleotide transhydrogenase [1–6], and pyruvate dehydrogenase complex in the nitrogen fixing process of the obligatory aerobic *Azotobacter vinelandii* was investigated.

As shown by Mortenson [7] pyruvate plays an important role in nitrogen fixation of anaerobically grown species, but until now no equivalent process has been found in aerobic bacteria.

This paper reports on some of the regulatory properties of PDC isolated and purified from *Azotobacter vinelandii* and the discovery of phosphotransacetylase (PTA) closely associated to the PDC. Furthermore the presence of acetate kinase was demonstrated in crude extracts. The possible significance of this discovery for nitrogen fixation is discussed.

2. Materials and methods

NAD^+, NADH, $NADP^+$, NADPH, TPP, acetyl CoA, bovine serum albumin, acetyl-phosphate, ADP and ATP were obtained from the Sigma Chemical Co. Phosphotransacetylase isolated from Clostridium, carnitine-acetyltransferase, citrate-synthase, hexokinase, glucose-6 phosphate dehydrogenase and CoA were obtained from Boehringer. Sepharose 4B was purchased from Pharmacia. All chemicals used were of analytical grade.

Abbreviations:

PDC,	pyruvate dehydrogenase complex;
PTA,	phosphotransacetylase;
DTNB,	5,5-dithiobis (2-nitrobenzoic acid);
TPP,	thiamine pyrophosphate;
GSH,	reduced glutathione.

Large-scale production of the bacteria on a nitrogen-free medium according to Pandit-Hovenkamp [8] was kindly performed by the Royal Netherlands Yeast and Fermentation Industries, Delft, The Netherlands, under the conditions described before [2]. Crude extracts were prepared as described earlier [2].

Isolation and purification of PDC was according to the methods of Reed and Willms [9], except that the iso-electric precipitation was omitted. Sometimes the purification procedure was a new one, involving polyethyleneglycol precipitation [10], leading to the same specific activities: 6–10 μmoles NADH/min/mg protein.

Activity measurements: PDC overall activity was measured according to Schwartz and Reed [11] in 50 mM phosphate buffer pH 7.0, 0.5 mM TPP, 5 mM $MgCl_2$, 2 mM GSH and the additions as indicated in the legend. In some experiments, as indicated, phosphate was replaced by 50 mM Tricine-KOH buffer pH 7.0. The activity of the dihydrolipoyl-transacetylase according to Schwartz and Reed [12]; PTA according to a modification [13] of the method of Bergmeyer; acetate kinase according to Rose [14]. All measurements were performed at 25° and the reaction started by the addition of enzyme. With the exception of acetate kinase initial velocities were measured.

CoA concentration was calculated from SH-group measurements according to Ellman [15]; acetyl CoA concentration was measured with a system containing oxaloacetate, citrate synthase and DTNB.

The CoA regenerating systems used consisted either of 0.2 mM oxaloacetate plus 3.5 enzyme units of citrate synthase or 20 mM carnitine plus 2 enzyme units of carnitine acetyltransferase.

3. Results and discussion

The influence of the pyruvate concentration on the overall reaction rate of the PDC complex with NAD^+ as final electron acceptor is shown in fig. 1. The K_m for pyruvate was found to be 0.8 mM; a Hill coefficient of $n = 1.7$ could be calculated for the pyruvate binding. These values are similar to those found for the PDC complex from *E. coli* [16–18], and it could be concluded that the pyruvate dehydrogenase component of the enzyme complex of *A. vinelandii*, like that of *E. coli*, contains two substrate binding sites.

Hansen and Henning [19] pointed out already that the inhibition of the PDC complex from *E. coli* by NADH is competitive with respect to NAD^+. The *A. vinelandii* PDC complex, as can be seen from fig. 2, behaves similarly. The apparent K_m for NAD^+, under the conditions of the assay, is 0.3 mM, and the K_I for NADH is 5 μM. The first value is about the same as the K_m-value found by Van den Broek [20] with pure lipoamide dehydrogenase from the same organism (0.18 mM).

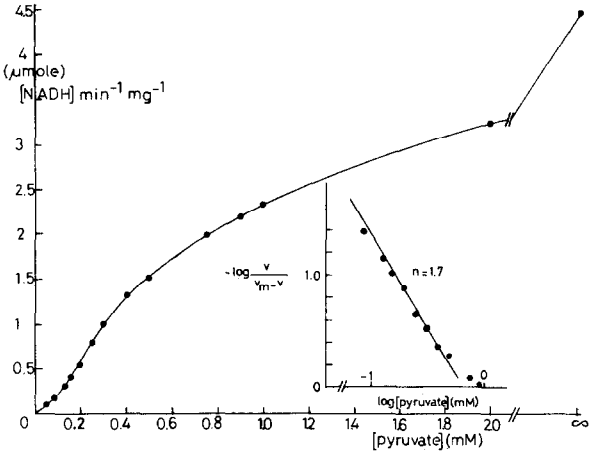

Fig. 1. Dependence of *Azotobacter* PDC overall activity on the pyruvate concentration. Conditions as mentioned under Materials and methods in the presence of 2 mM NAD^+, 0.13 mM CoA and the pyruvate concentration as indicated. Insert: Hill plot derived from these results.

Like in PDC from *E. coli* [17], acetyl CoA is a competitive inhibitor with respect to pyruvate in the *Azotobacter* complex. In the pyruvate decarboxylase assay [12], in which Tricine buffer was replaced by phosphate buffer linear Lineweaver-Burk plots were obtained from which was calculated: K_m (pyruvate) = 140 μM and K_I (acetyl CoA) = 40 μM. When on the other hand the influence of acetyl CoA on the overall activity of PDC was assayed in phosphate buffer (fig. 3), the inhibition pattern showed cooperativity, Hill coefficient $n = 1.6$; the concentration of acetyl CoA giving 50% inhibition was 30 μM. When, however, phosphate buffer was replaced by Tricine buffer, this cooperativity disappeared, the Hill coefficient $n \approx 1.0$, while the degree of inhibition was much higher ($K_I \approx 8$ μM). This latter inhibition pattern is similar to that observed by Shen and Atkinson with PDC from *E. coli* [21].

The following experiments provide an explanation for this observation by demonstrating that the delayed inhibition by acetyl CoA, as indicated by $n = 1.6$, is due to the (partial) conversion of acetyl CoA into CoA. When the overall activity of the complex is followed by varying the CoA concentration, at very low CoA concentrations, a sigmoid velocity–CoA curve is observed (fig. 4). The shape of the curve is not due to cooperative binding of the CoA as is shown by the rectangular hyperbola observed in the presence of a CoA-regenerating system. This effect does not depend on

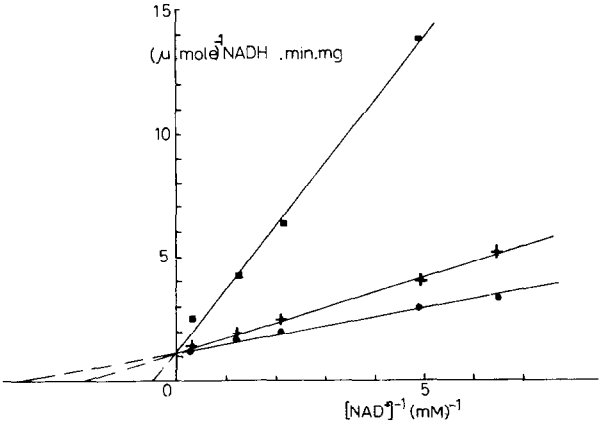

Fig. 2. Effect of NADH on the reduction of NAD^+ in the overall reaction of *Azotobacter* PDC. Conditions as mentioned under Materials and methods, in the presence of 5 mM pyruvate, 0.13 mM CoA and the NAD^+ concentrations as indicated. (●–●–●): no NADH; (+–+–+): 0.85 μM NADH; (■–■–■): 10.26 μM NADH.

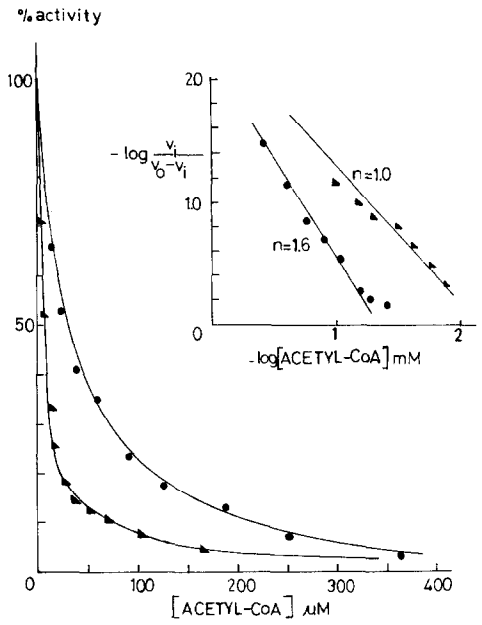

Fig. 3. Effect of acetyl CoA on the overall activity of *Azotobacter* PDC in the presence and absence of phosphate. Conditions as described under Materials and methods in the presence of 2 mM NAD^+, 0.16 mM pyruvate, 0.11 mM CoA and the concentrations of acetyl CoA as indicated. (●–●–●): phosphate buffer; (▲–▲–▲): Tricine–KOH buffer. Insert: Hill plots derived from these results.

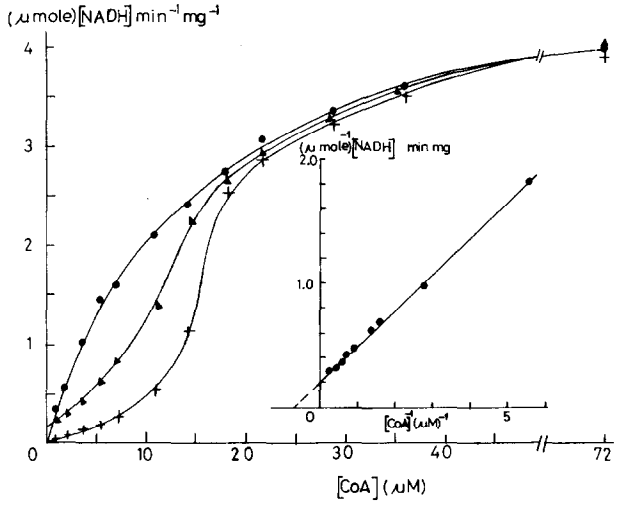

Fig. 4. Influence of CoA on the overall activity of *Azotobacter* PDC. Reaction mixture as mentioned under Materials and methods, in the presence of 5 mM pyruvate, 2 mM NAD^+ and the CoA concentrations indicated. (+–+–+): no regenerating systems present (▲–▲–▲): no regenerating system but in the presence of 12 μM acetyl CoA; (●–●–●): in the presence of a regenerating system. Insert: reciprocal plot derived from v versus CoA curve in the presence of a regenerating system.

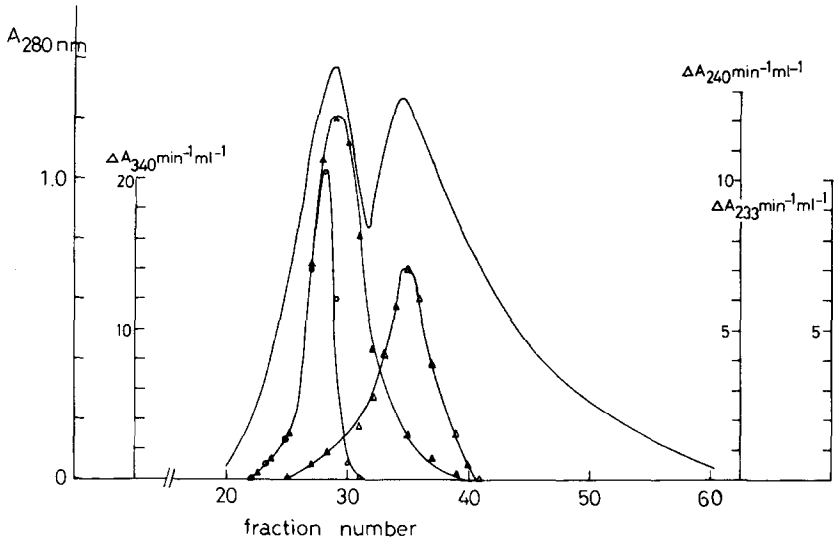

Fig. 5. Elution pattern obtained upon chromatography of *Azotobacter* PDC preparations (specific activity 6 μmoles × min^{-1} × mg^{-1}) on Sepharose 4B. 80 mg of enzyme preparation was applied to a column of 2 × 80 cm. Elution was performed with 0.1 M potassium phosphate buffer pH 7.0 at a flow rate of 9 ml per hr. Fractions of 5 ml were collected. Conditions for assay measured under Materials and methods. (——): A_{280} nm; (○–○–○): PDC activity, measured at 340 nm, in the presence of 2 mM NAD^+, 0.13 mM CoA and 5 mM pyruvate; (▲–▲–▲): dihydrolipoyl transacetylase activity, measured at 240 nm; (△–△–△): PTA activity, measured at 233 nm.

the kind of CoA-regenerating system used. The cooperative effect in the absence of a CoA-regenerating system thus must be due to the formation of small amounts of acetyl CoA, which inhibit the reaction effectively due to the low K_I. When, however, acetyl CoA is added to the system without a CoA regenerating sytem activation is observed rather than inhibition. A similar experiment performed in the absence of phosphate in Tricine buffer shows that acetyl CoA inhibits the reaction.

It cannot be concluded that the main inhibitory effect of acetyl CoA is on the CoA-site (K_I = 8 μM) rather than on the pyruvate site (K_I = 40 μM), because the experiments show that acetyl CoA is rapidly converted by a phosphate-dependent reaction into CoA.

It is likely that acetyl CoA is converted into acetylphosphate. Fig. 5 shows that phosphotransacetylase (PTA) is present in PDC. Control experiments show that this activity is due to a separate enzyme. In view of these observations we suggest that the activity-stimulating effect of inorganic phosphate on acetyl CoA-inhibited PDC from *E. coli* [11] is, as in the case of the *Azotobacter* complex, due to PTA activity, demonstrated [13] to be present in this facultative anaerobic bacterium.

On the basis of the close association between PTA and PDC during several purification steps involving ammonium sulphate precipitation, polyethylene glycol precipitation, adsorption chromatography, gel filtration, sucrose density gradient centrifugation, iso-electric precipitation, independent of whether the crude extracts were prepared by sonication or by osmotic shock, we suggest a functional relation between these enzymes. In addition we have been able to demonstrate that acetate kinase activity is present in crude extracts.

The cooperativity of CoA on the overall reaction of PDC is clearly due to a subtle mechanism of product inhibition by acetyl CoA and subsequent conversion of acetyl CoA into acetylphosphate. Considering the unfavourable equilibrium of the acetate kinase reaction towards acetylphosphate formation [22] it is clear that the purpose of this "soluble" system is directed towards ATP synthesis coupled to non-particle bound pyruvate oxidation.

Indeed we have been able to demonstrate in crude extracts the anaerobic formation of ATP from pyruvate and ADP in the presence of appropriate cofactors. Furthermore we succeeded to use acetylphosphate via the acetate kinase reaction as energy-donor in the reduction of N_2 to NH_3 [22].

We therefore conclude that this soluble PDC being in close interaction with pyridine nucleotide transhydrogenase, PTA and acetate kinase is the main donor of reducing equivalents and high-energy phosphate in the anaerobic process of reducing nitrogen in this aerobic bacterium.

Acknowledgements

The investigations were subsidized by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.). The authors wish to thank Mr. P.P. van der Puyl for skillfull technical assistance. We are very much indebted to Dr. J. de Flines from the Royal Netherlands Yeast and Fermentation Industries (Delft, The Netherlands) for the large-scale production of the *Azotobacter* strain.

References

[1] H.W.J. van den Broek and C. Veeger, FEBS Letters 1 (1968) 301.
[2] H.W.J. van den Broek, J.S. Santema, J.H. Wassink and C. Veeger, European J. Biochem. 24 (1971) 31.
[3] H.W.J. van den Broek, J.F.L. van Breemen, E.F.J. van Bruggen and C. Veeger, European J. Biochem. 24 (1971) 46.
[4] H.W.J. van den Broek, J.S. Santema and C. Veeger, European J. Biochem. 24 (1971) 55.
[5] H.W.J. van den Broek and C. Veeger, European J. Biochem. 24 (1971) 63.
[6] H.W.J. van den Broek and C. Veeger, European J. Biochem. 24 (1971) 72.
[7] L.E. Mortenson, R.C. Valentine and J.E. Carnahan, J. Biol. Chem. 238 (1962) 794.
[8] H.G. Pandit-Hovenkamp, in: Oxidation and Phosphorylation, eds. R.W. Estabrook and M.E. Pullman (Academic Press, New York, 1966).
[9] L.J. Reed and C.R. Willms, Methods Enzymol. 9 (1965) 247.
[10] J. Krul and C. Veeger, to be published.
[11] E.R. Schwartz and L.J. Reed, Biochemistry 9 (1970) 1434.
[12] E.R. Schwartz and L.J. Reed, J. Biol. Chem. 244 (1969) 6074.
[13] M. Shimuzu, T. Suzuki, K. Kameda and Y. Abiko, Biochim. Biophys. Acta 191 (1969) 550.
[14] I.A. Rose, Methods Enzymol. 1 (1955) 591.
[15] G.L. Ellman, Arch. Biochem. Biophys. 82 (1959) 70.
[16] H. Bisswanger and U. Henning, European J. Biochem. 24 (1971) 376.

[17] E.R. Schwartz, L.O. Old and L.J. Reed, Biochem. Biophys Res. Commun. 31 (1968) 495.
[18] E.R. Schwartz and L.J. Reed, Federation Proc. 27 (1968) 389.
[19] R.G. Hansen and U. Henning, Biochim. Biophys. Acta 122 (1966) 355.
[20] H.W.J. van den Broek, Ph.D. Thesis, Mededelingen Landbouwhogeschool 71-8 (1971), Landbouwhogeschool, Wageningen, The Netherlands.
[21] L.C. Shen and D.E. Atkinson, J. Biol. Chem. 245 (1970) 5974.
[22] I.A. Rose, M. Grunberg-Manago, S.R. Korey and S. Ochoa, J. Biol. Chem. 211 (1954) 737.
[23] H.B.C.M. Haaker and C. Veeger, to be published.