TRANSFORMATION OF THE 4-COMPONENT PYRUVATE DEHYDROGENASE COMPLEX FROM *AZOTOBACTER VINELANDII* INTO A 3-COMPONENT COMPLEX

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1. Introduction

Blue dextran-Sepharose has been used with success as a matrix for affinity chromatography of proteins which contain the so-called dinucleotide fold [1–4]. In some other proteins the affinity seems to be due to ionic or $\pi-\pi$ interactions [1,5]. This matrix seems very suitable for affinity chromatography of keto-acid dehydrogenase complexes because their activities are NAD^+-dependent and regulated by a variety of nucleotides.

In contrast to other bacterial keto-acid dehydrogenase complexes, the pyruvate dehydrogenase complex from *Azotobacter vinelandii* contains a fourth component with transacetylase activity [6]. We have called this component, low molecular weight lipoyl-transacetylase, to differentiate it from the transacetylase usually present, here called, high molecular weight lipoyl-transacetylase. This fourth component is co-purified in a constant ratio to the other components of the complex and is an active constituent of the complex, i.e., it does accept the acetyl group at relatively high pyruvate concentrations [7,8]. Crosslinking experiments [8] with diimidates have shown that the low molecular weight lipoyl-transacetylase interacts with the pyruvate dehydrogenase component of the complex.

Here we present results describing the behaviour of the pyruvate dehydrogenase complexes from *Az. vinelandii* and *Escherichia coli* Crookes on blue dextrane–Sepharose 4B columns. It is shown that the 4-component complex from *Az. vinelandii* binds strongly to the column through its low molecular weight lipoyl-transacetylase. Upon elution with 0.6 M KCl, a 'normal' 3-component complex is obtained. Some of the properties of this complex are described.

2. Materials and methods

The pyruvate dehydrogenase complex from *Az. vinelandii* (ATCC 478) was prepared according to Bresters et al [6]. The pyruvate dehydrogenase complex from *E. coli* Crookes was prepared as described by Eley et al. [9]. Both complexes were purified and stored in the presence of EDTA and *p*-phenylmethane sulfonylfluoride. Sepharose 4B and blue dextran were obtained from Pharmacia. Blue dextran was covalently coupled to activated Sepharose 4B using the procedure described by Ryan and Vestling [10]. A blue dextran-Sepharose 4B column (dimension 1.0 × 5 cm, void vol. approx. 8 ml) was used. The column was equilibrated with 50 mM potassium phosphate buffer, pH 7.0. About 15 mg pyruvate dehydrogenase complex in 0.5 ml was applied to the column. The column was washed with 50 mM phosphate buffer, pH 7.0, until protein absorbance and enzyme activity were no longer detectable in the effluents (usually 80 ml) and then eluted with 0.6 M potassium chloride in 50 mM phosphate buffer pH 7.0.

Pyruvate-NAD^+ reductase (overall) activity, pyruvate-$K_3Fe(CN)_6$ reductase (pyruvate dehydrogenase) activity, reduced lipoate-CoA transacetylase activity and lipoamide dehydrogenase activity were assayed as described earlier [6,11–13].

Sedimentation and diffusion experiments were performed with an MSE analytical ultracentrifuge.

The sedimentation runs were carried out in 50 mM potassium phosphate buffer, pH 7.0, at 20°C. Scans were made with time intervals of 360 s with rotor speeds of 30 000 rev/min and 35 000 rev/min for sedimentation experiments and time intervals of 900 s after reaching equilibrium with a rotor speed of 2650 rev/min for diffusion experiments. The sedimentation and diffusion coefficients were calculated according to Elias [14]. The molecular weights were calculated from sedimentation coefficients and diffusion coefficients assuming partial specific vol. 0.75 $ml.g^{-1}$.

Sodium dodecylsulphate–polyacrylamide gel electrophoresis was performed according to Laemmli [15].

3. Results and discussion

3.1. *Behaviour of the complexes from* Az. vinelandii *and* E.coli *on the blue dextran–Sepharose 4B column*

The complex from *Az. vinelandii* binds strongly to the blue dextran-Sepharose 4B column, equilibrated with 50 mM potassium phosphate buffer pH 7.0. It elutes as a sharp peak with 0.6 M potassium chloride (fig.1). A 9.1 mg yield is obtained from 15 mg pyruvate dehydrogenase complex. The complex eluted from the column consists of pyruvate dehydrogenase, high molecular weight lipoyl-transacetylase and lipoamide dehydrogenase as revealed by sodium dodecylsulphate–gel electrophoresis. In none of the fractions could the low molecular weight lipoyl-transacetylase be detected. The low molecular weight lipoyl-transacetylase remains bound to the column even after elution with 2.5 M KCl. A fraction containing pyruvate dehydrogenase and low molecular weight lipoyl-transacetylase was eluted when 5% Triton X-100 was applied to the column after elution with 0.6 M potassium chloride. In this fraction only transacetylase activity could be measured. Apparently the 4-component complex is bound to the blue dextran through the low molecular weight lipoyl-transacetylase component. At high salt concentrations interactions between this component and other components of the complex are weakened. Because no appreciable elution was seen with nucleotides such as NAD^+, CoA or AMP, binding is probably not due to the presence of a dinucleotide fold in the low-molecular weight lipoyl-transacetylase but rather to an ionic or π–π type of interaction with the dye. The low molecular weight lipoyl-transacetylase has a very high content of aromatic amino acid residues as can be concluded from its ultraviolet absorption. Upon

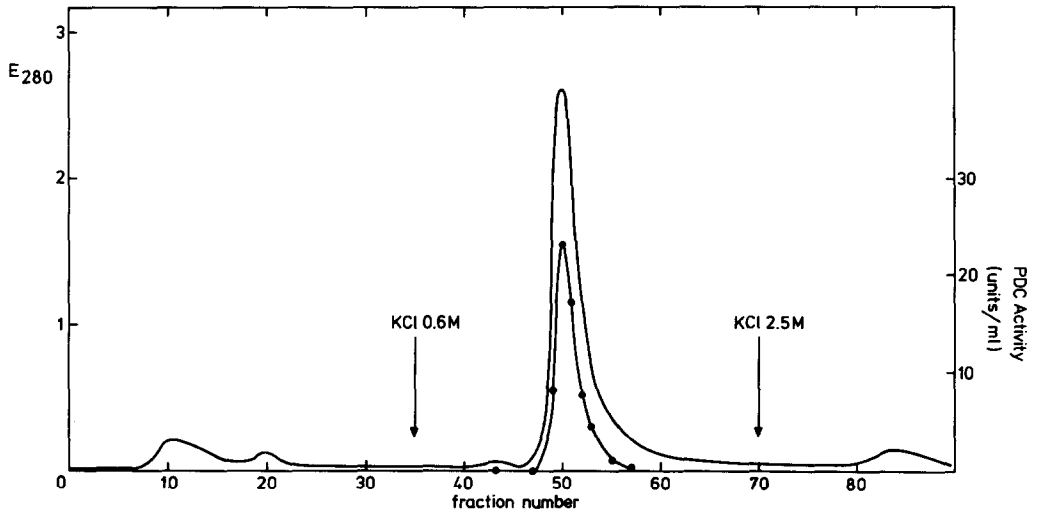

Fig.1. Elution pattern of *Az. vinelandii* pyruvate dehydrogenase complex (15 mg) on a blue dextran–Sepharose 4B column. The complex was eluted as described in Materials and methods. Fractions of 2.5 ml were collected at an elution rate of 30 ml/h. (——) Absorption at 280 nm; (–•–•–) Pyruvate-NAD^+ reductase activity in μmol $NADH.min^{-1}.ml^{-1}$.

rechromatographing the eluted complex, after dialysis against 50 mM potassium phosphate buffer pH 7.0, under identical conditions, more than 90% of the activity appears in the void volume. The pyruvate dehydrogenase complex from *E. coli* Crookes also elutes in the void volume, even in buffers of low ionic strength. A small part of the complex, about 10% of the activity is bound and not eluted below a concentration of 0.6 M KCl. A protein with mol. wt 63 000, often found in the complex isolated according to Eley et al. [9] was removed by this procedure, but the complex in the void still contained this protein.

3.2. *Properties of the three component complex from* Az. vinelandii

The values for the overall activities and the partial activities of the 3-component complex and of the original 4-component complex, are given in table 1.

The removal of the low molecular weight lipoyl-transacetylase and part of the pyruvate dehydrogenase apparently has no effect on the overall reaction. Partial removal of the pyruvate dehydrogenase from the *E. coli* complex was also reported not to influence the overall activity [16]. In this case there is a clear connection between the 'excess' pyruvate dehydrogenase and the low molecular weight lipoyl-transacetylase. It may well be that such a connection also exists in the *E. coli* complex, but has hitherto escaped attention.

The slight increase in lipoyl-transacetylase activity of the 3-component complex indicates that the contribution of the low molecular weight lipoyl-transacetylase to the total activity is probably very low. The total activity in the Triton X-100 eluate is only 5.5 units. No inactivation of the transacetylase has been observed during the time period necessary for the experiment. Because we found that the low molecular weight lipoyl-transacetylase is rapidly acetylated [7] this suggests that the physiological acetyl acceptor might be different from CoA or alternatively that the enzyme reacts poorly with CoASAc in the assay. The FAD content of the 3-component complex (table 2) has increased and is comparable with that of the *E. coli* complex [12,17]. A 3-fold increase in lipoamide dehydrogenase activity of the 3-component complex is found which is much larger than the increase in FAD-content. A further 4-fold increase is found in the pure enzyme [18]. This again indicates a functional association of the low molecular weight lipoyl-transacetylase with the complex. This could be due to an increased accessibility of the substrate lipoamide, which is of no significance for the overall reaction. Alkonyi et al. [19] observed that the unresolved lipoamide dehydrogenase of the pigeon breast-muscle pyruvate dehydrogenase complex is completely inactive in the NADH: lipoamide reductase assay, in contrast to the resolved enzyme.

From the sedimentation and diffusion coefficients, mol. wt 760 000 was calculated for the 3-component complex, which is in good agreement with the molecular weight calculated from the FAD content assuming lipoamide dehydrogenase to be a dimer.

Table 1

Partial and overall activities of 4- and 3-component complexes from *Az. vinelandii*, before and after blue dextran–Sepharose chromatography, respectively

Enzyme	4-Component complex			3-Component complex		
	(Units/mg protein)	(Units/μmol FAD)	Total units	Units/mg protein	Units/μmol FAD	Total units
Pyruvate-NAD^+ reductase	7.0	4100	105	12.0	4450	109
Pyruvate-$Fe(CN)_6$ reductase	0.12	70	1.8	0.13	40	1.2
Lipoyl-trans-acetylase	3.4	2000	51	7.4	2700	67
Lipoamide dehydrogenase	1.9	1100	28.5	9.5	3500	86.5

Table 2
Comparison of some physical constants of the two pyruvate dehydrogenase complexes from *Az. vinelandii*

Physical constants	4-Component pyruvate dehydrogenase complex from *Az. vinelandii*	3-Component pyruvate dehydrogenase complex from *Az. vinelandii*
$s_{20,w}$	19 S [6] (protein conc. > 0.4 mg/ml) 16 S (protein conc. < 0.2 mg/ml)	15 S (conc. 0.7 mg protein/ml) 14 S (conc. 0.2 mg/ml)
$D_{20,w}$	1.96×10^{-7} cm^2.s^{-1} [6]	1.90×10^{-7} cm^2.s^{-1}
Mol. wt (from *s*- and *D*-values and $\bar{\nu}$ 0.75)	970 000 [6]	760 000 (15 S)
FAD content	1.77 nmol FAD/mg protein	2.6–2.8 nmol/mg protein
Mol. wt (calculated from FAD-content)[a]	1 130 000	740 000

[a] It was assumed that lipoamide dehydrogenase is present as a dimer in the complex

Other properties of the 3-component complex, such as the Hill coefficient for the overall reaction, the stimulation by AMP and the inhibition by CoASAc were not different from the original complex [20].

The results indicate that the low molecular weight lipoyl-transacetylase and part of the pyruvate dehydrogenase form a separate entity within the complex, the function of which will now be investigated.

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