Binding and dissociation of the pyruvate dehydrogenase complex of *Azotobacter vinelandii* on thiol–Sepharose

Adriana C. de Graaf-Hess and Arie de Kok

Agricultural University, Wageningen, The Netherlands

Received 26 April 1982

1. INTRODUCTION

Activated thiol–Sepharose 4B, a mixed disulphide of 2,2′-dipyridyl disulphide and glutathione coupled to CNBr-activated Sepharose 4B, has been developed to couple covalently thiol-containing proteins [1,2]. Thus this matrix seems suitable for specific binding of those enzymes which do not contain reactive SH groups as such, but in which accessable SH groups can be generated by the enzymatic reaction. Examples of such enzymes are the 2-oxoacid dehydrogenase complexes where incubation with the 2-oxoacid substrate generates enzyme-bound dihydrolipoylgroups. Specific binding of these enzymes to thiol–sepharose is not only of interest for the purification but also for the dissociation and purification of the components of these multi-enzyme complexes.

Like that of other prokaryotes, the pyruvate dehydrogenase complex of *Azotobacter vinelandii* consists of 3 types of subunits, pyruvate dehydrogenase (E_1), lipoyltransacetylase (E_2) and lipoamide dehydrogenase (E3). Compared to complexes from other sources the size of the *A. vinelandii* complex is small, 19 S, and it contains only 10–14 subunits [3]. Dissociation of the complex by classical methods such as high pH [4], high salt concentration [5] or urea [4,5] and subsequent purification of the components by hydroxylapatite chromatography [4,5] or gel filtration [6] were unsuccessful, partly because of incomplete dissociation and inactivation during the lengthy procedures, partly because of the relatively small M_r differences of the components and subcomplexes obtained.

This paper describes the immobilization of the pyruvate dehydrogenase complex on thiol–Sepharose after reductive acetylation of the lipoyl-groups in the presence of pyruvate and the subsequent elution of the complex with hydroxylamine. This method was used to obtain the pure components of the complex in high yield in a one step procedure.

2. MATERIALS AND METHODS

The pyruvate dehydrogenase complex from *A. vinelandii* cells was prepared according to [7] (procedure III). Pyruvate-NAD^+ reductase (overall) activity, pyruvate-$K_3Fe(CN)6$ reductase (E_1) activity, acetyl-CoA-reduced lipoate transacetylase (E_2) activity and NADH-lipoate reductase (E_3) activity were assayed as in [8–11]. A molar extinction coefficient of 4400 $M^{-1} \cdot cm^{-1}$ at 240 nm was used for acetyldihydrolipoate [12].

Thiol–Sepharose 4B was from Pharmacia. A column (9 × 1.6 cm) was equilibrated with 50 mM phosphate buffer (pH 7.0), 0.3 M NaCl (buffer A) containing 10 mM pyruvate. The complex (40 mg) was slowly applied to the column, which was then washed with buffer A at 10 ml/h. A small amount of complex, 5–10% based on overall activity, was usually eluted in this stage. The complex was eluted with buffer in which NaCl was replaced by 0.5 M hydroxylamine (pH 7.0) and prepared freshly.

In order to remove unreacted 2-thiopyridone which obscures the protein absorbance, the column can be washed with buffer A contining 20 mM cysteine (pH 7.0) at which pH the complex is not removed. For dissociation purposes, after binding of the complex and removal of 2-thiopyridone and

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

cysteine, E_1, E_3 and E_2 were eluted (at 30 ml/h) in this order with, respectively, buffer A brought to pH 8.8 with concentrated ethanolamine, buffer A containing 3 M KBr and buffer A in which 0.3 M NaCl was substituted for 0.5 M hydroxylamine (pH 7.0). Between buffer changes the column was washed with buffer A. Fractions, containing E_1 were neutralized with 1 M KH_2PO_4. Immediately after elution all components were dialyzed against 50 mM phosphate, 1 mM EDTA (pH 7.0). For purification purposes, thiol-containing proteins were first removed by passage through thiol–Sepharose in the absence of pyruvate. The eluate, containing the complex, was then absorbed on another column and eluted by the above procedure.

SDS–polyacrylamide gel electrophoresis was performed according to [13] as modified [14]. Regeneration of thiol–Sepharose was performed as in [1]. Care was taken to remove hydroxylamine directly after the elution procedure, because prolonged contact led to loss of the glutathione spacer arm.

Protein was determined according to [15] after dialysis of the fractions against 50 mM phosphate buffer or by the $E^{1\%}_{280}$ values of 14.3, 6.3 and 9.2 for E_1, E_2 and E_3, respectively.

3. RESULTS AND DISCUSSION

Unreacted pyruvate dehydrogenase complex in buffer A passes without retardation through the column. This indicates that the complex contains no reactive SH groups on the surface. Reductive acetylation of the lipoyl groups resulted in almost complete binding (> 90%). With some preparations addition of Mg^{2+} and TPP was necessary to obtain maximum binding, whereas EDTA in the binding buffer decreased the binding capacity.

The specific activity of the complex in the wash was usually low, thus this may represent an inactive fraction of the original preparation. It is remarkable that the partial activities of this inactivated complex (table 1) are normal, which indicates the lipoyl-group as the site of inactivation. It is clear that the lipoyl SH groups, bound to the E_2 substructure, protrude to the outside of the complex or, at least, are easily accessible to the glutathione spacer arm.

Elution of the complex with 20 mM cysteine was strongly pH-dependent. At pH 7.0, the remaining 2-pyridone groups were eluted, but none of the complex. At pH 8.0, the complex was slowly eluted, and inactivation was observed. Because this could be due to instability of the complex in the acetylated form [16] another elution procedure was preferred. Rapid elution of fully active complex was accomplished with 0.5 M hydroxylamine at pH 7.0. This nucleophile results in rapid deacetylation, thus generating in situ a SH group, which could bring about cleavage of the neighbouring S–S bridge with the matrix resulting in the formation of oxidized lipoate and reduced spacer arm. This mechanism has its analogy to the proposed mechanism [17]

Table 1

Partial and overall activities of the pyruvate dehydrogenase complex and its components obtained after dissociation on thiol–Sepharose

	Pyruvate-NAD^+ reductase (overall activity)		Pyruvate-$Fe(CN)^6$ reductase (E_1 activity)		AcetylCoA-di-hydrolipoyl transacetylase (E_2 activity)		NADH-lipoamide reductase (E_3 activity)	
	Total units	Units /mg	Total units	Units /mg	Total units	Units /mg	Total units	Units /mg
Complex	535	12.8	11	0.26	131	3.1	169	4.0
Wash	13.8	2.9	1.3	0.28	11	2.3	17.4	3.7
pH 8.8 fraction	0		9.7	0.68	0		0	
3 M KBr fraction	0		0		0		141.6	34.6
0.5 M NH_2OH fraction	0		0		116.2	12.6	0	

for the lipoate-catalysed cleavage of 5,5′-dithiobis(2-nitrobenzoate) (TNBS) by NADH in the presence of lipoamide dehydrogenase:

$$\text{lip}\begin{matrix}\diagup \text{S} \\ \diagdown \text{S}\end{matrix} + \text{NADH} + \text{H}^+ \overset{E_3}{\rightleftharpoons} \text{lip}\begin{matrix}\diagup \text{SH} \\ \diagdown \text{SH}\end{matrix} + \text{NAD}^+$$

$$\text{lip}\begin{matrix}\diagup \text{SH} \\ \diagdown \text{SH}\end{matrix} + \text{TNBS} \rightarrow \text{lip}\begin{matrix}\diagup \text{S-TNB} \\ \diagdown \text{SH}\end{matrix} + \text{TNB}^- + \text{H}^+$$

$$\text{lip}\begin{matrix}\diagup \text{S-TNB} \\ \diagdown \text{SH}\end{matrix} \rightarrow \text{lip}\begin{matrix}\diagup \text{S} \\ \diagdown \text{S}\end{matrix} + \text{TNB}^- + \text{H}^+$$

In accordance with this scheme no binding to thiol–Sepharose was observed when the complex was reduced with NADH instead of pyruvate. Although this method should be suitable for purification of the complex, we were not successful in its application.

With impure preparations, such as cell-free extract, most of the enzyme passes through the column. This was not due to competitive binding of other thiol-containing proteins, because it made no difference when the preparation was first applied to thiol–Sepharose in the absence of pyruvate. A possible explanation could be deacetylation during the relatively long time required to bind the dilute enzyme solution to the column [16]. Prolonged standing (> 30 min) of the complex in the acetylated form leads to inactivation, especially under aerobic conditions. However, bound to the matrix no inactivation has been observed for 2 days.

With the concentrated pure preparations 5–10% of the complex passes directly through the column. Though it cannot be excluded that some deacetylation or inactivation occurs during the binding procedure, the eluted complex has a 5–10% increased specific activity, which indicates removal of pre-existent inactive material. In this respect, the method can be used to separate active from inactive complex as in the case of papain [1].

Fig.1 shows the results of a typical dissociation experiment. To observe protein absorbance at 280 nm, all unreacted 2-pyridone groups were removed by washing the column with 20 mM cysteine (pH 7.0). Otherwise some of these groups are eluted at high pH and especially by hydroxylamine. A pH of 8.8 is high enough to elute immediately the bulk of the E_1 component. Elution should be continued rather long (at least 3 column volumes) to elute all

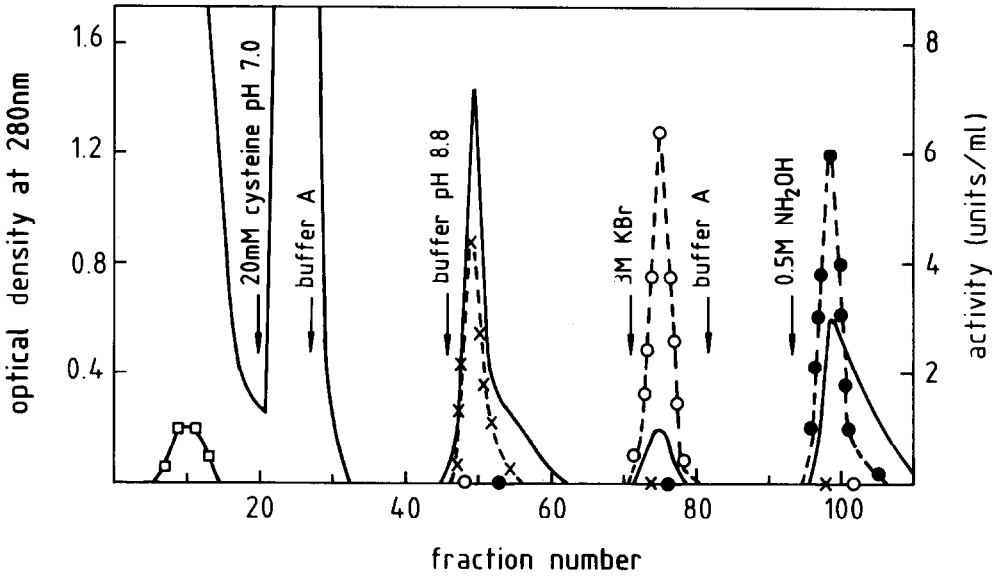

Fig.1. Stepwise elution of the components of the pyruvate dehydrogenase complex on thiol–Sepharose; conditions were as in section 2. Fractions of 4.0 ml were collected at an elution rate of 30 ml/h; (——) absorption at 280 nm; (-□-□-) complex activity; (-×-×) E_1 activity × 10; (-○-○-) E_3 activity; (-●-●-) E_2 activity.

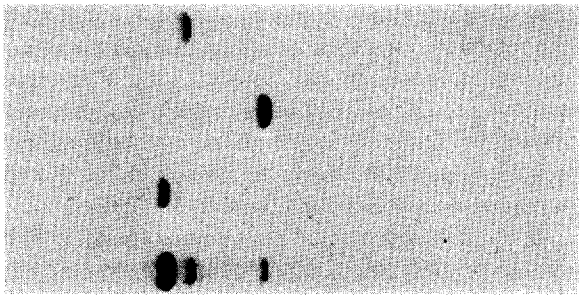

Fig.2. SDS–PAGE patterns of complex and pooled fractions of the elution peaks of the components as indicated in fig.1: lane 1, pyruvate dehydrogenase complex; lane 2, pyruvate dehydrogenase; lane 3, lipoamide dehydrogenase and lane 4, lipoyltransacetylase.

of the E_1 component. A pH of 9.7 can be used to shorten this procedure but this leads to losses in E_2 activity. E_3 is subsequently eluted with 3 M KBr. Though 3 M KBr has been used to remove FAD from the holo enzyme, we have no indication that apoenzyme is formed. Addition of FAD after elution has no effect and the sum of the activities of E_3 in wash and eluate is close to 100% (see table 1). Finally, E_2 is eluted with 0.5 M hydroxylamine (pH 7.0). The components were pure on SDS gels (fig.2). The increase in specific activity is to be expected from the weight fractions of the components in the complex [7]. The only exception is E_1 where the increase in specific activity in somewhat higher than the expected factor 1.7 based on a E_1 content of 60%. Preliminary experiments have shown that the components are fully active in reconstitution of complex activity. Details of these experiments will be described elsewhere.

For the first time a short and elegant method is described to obtain the components of the 2-oxoacid dehydrogenase complex in an essentially one step procedure. Dissociation occurs under milder conditions as compared to methods used so far, leading to higher yields. This method seems in principle suitable for the dissociation of other 2-oxoacid dehydrogenase complexes as well.

ACKNOWLEDGEMENTS

The present investigation was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organisation for the Advancement of Pure Research (ZWO).

REFERENCES

[1] Brocklehurst, K., Carlsson, J. and Kierstan H.P.J. (1973) Biochem. J. 133, 573–584.
[2] Carlsson, J. and Svensson, A. (1974) FEBS Lett. 42, 183–186.
[3] De Abreu, R.A., De Kok, A. and Veeger, C. (1977) FEBS Lett. 82, 89–92.
[4] Koïke, M., Reed, L.J. and Caroll, W.R. (1963), J. Biol. Chem. 238, 30–39.
[5] Kresze, G.B. and Ronft, H. (1981) Eur. J. Biochem. 119, 581–587.
[6] Coggins, J.A., Hooper, E.A. and Perham, R.N. (1976) Biochemistry 15, 2527–2532.
[7] Bosma, H.J., De Graaf-Hess, A.C., De Kok, A., Veeger, C., Visser, A.J.W.G. and Voordouw, G. (1982) Int. Thiamine Conf., Ann. NY Acad. Sci. 378, 265–287.
[8] Schwarz, E.R. and Reed, L.J. (1969) J. Biol. Chem. 244, 6074–6079.
[9] Schwarz, E.R. and Reed, L.J. (1970) Biochemistry 9, 1434–1439.
[10] Massey, V. and Veeger, C. (1961) Biochim. Biophys. Acta 48, 33–47.
[11] Bresters, T.W., De Abreu, R.A., De Kok, A., Visser, J. and Veeger, C. (1975) Eur. J. Biochem. 59, 335–345.
[12] Daigo, K. and Reed, L.J. (1962) J.Am.Chem.Soc. 84, 659–662.
[13] Laemli, U.K. (1970) Nature 227, 680–685.
[14] Dorssers, L., Zabel, P., Van der Meer, J. and Van Kammen, A. (1982) Virology 116, 236–249.
[15] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J.Biol.Chem. 193, 265–275.
[16] De Abreu, R.A., De Kok A., De Graaf-Hess, A.C. and Veeger, C. (1977) Eur.J.Biochem. 81, 357–364.
[17] Brown, J.B. and Perham, R.N. (1976) Biochem.J. 155, 419–427.