ELUTION OF PYRUVATE KINASE FROM CM-CELLULOSE COLUMNS BY ITS ALLOSTERIC EFFECTOR. A NOVEL METHOD FOR ENZYME PURIFICATION

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Received 14 July 1969
Revised version received 25 July 1969

1. Introduction

In the last decade there has been considerable interest in the possibility of utilizing the specificity of enzyme-substrate interaction for enzyme purification [1–5]. This principle has been successfully applied by Pogell [6] for selective substrate elution of fructose diphosphatase from CM-cellulose columns. Recently different procedures based on affinity chromatography have also been reported [7–9].

It is now accepted that allosteric proteins have two or more stereospecifically different non-overlapping binding sites [10]. This property should enable one to devise new procedures for purifying regulatory enzymes.

In this report the potential application of specific effector-enzyme interactions is illustrated by the purification of liver pyruvate kinase "type L" on CM-cellulose columns, using, as eluent, dilute solutions of fructose 1,6-diphosphate (FDP), the positive allosteric effector [11–15].

2. Methods

The enzymes were isolated from rat liver as already described [16]. After ammonium sulfate precipitation, the extract was passed through a Sephadex G-25 column equilibrated with 5 mM Tris-maleate buffer pH 6.0 containing 0.5 mM EDTA and 1 mM dithiothreitol (Calbiochem, USA). Such preparations were applied to columns (12×100 mm) of CM-cellulose (Bio-Rad Laboratory, California, USA) previously washed according to Peterson and Sober [17] and equilibrated with the same mixture. First 50 ml of the above-mentioned buffer were passed through the column. The enzyme was then eluted with the same solution containing the specific eluent being tested also at pH 6.0. Careful control of the pH of all the solutions is necessary to prevent erratic behavior of the ion exchanger with the enzyme.

Pyruvate kinase activity was measured by the colorimetric assay procedure previously described [12]. One unit of enzyme is defined as the amount which catalyzes the formation of 1 μmole of pyruvate per min under the conditions of the standard assay. Fructose diphosphatase activity was assayed according to Pontremoli et al. [18]. Protein was determined by the method of Lowry et al. [19].

3. Results and discussion

A typical elution pattern from chromatography of a partially purified enzyme on a CM-cellulose column is shown in fig. 1. A large amount of protein was readily washed with the equilibrating buffer. Pyruvate kinase was retained and could be eluted with the same buffer but containing 0.5 mM of FDP. The recovery of the enzyme obtained by chromatography on CM-cellulose was 60% and the overall purification was 30-fold. A 50-fold purification could be obtained by selecting the material eluting at the center of the peak.

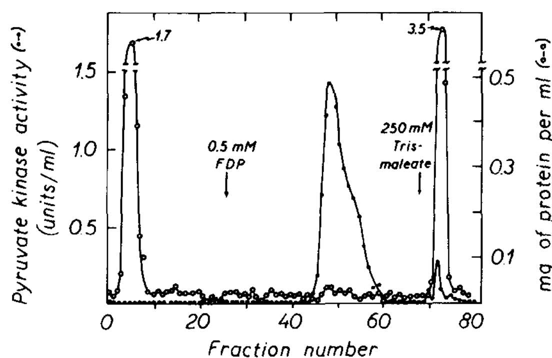

Fig. 1. Elution profile of pyruvate kinase from CM-cellulose column chromatography. The preparation poured over the column contained 15 mg of protein with a specific activity of 1.1 units/mg. Two-milliliter fractions were collected, the flow rate was about 2 ml per min and the experiments were performed at 4–6°C. For other conditions of the experiment see section 2.

Further elution with 250 mM Tris-maleate, pH 6.0, removed more protein but no activity. In fig. 1 it is possible to see that the enzyme activity peak is slightly asymmetric. Although this could indicate that more than one form of the enzyme is being eluted, this shoulder in the elution pattern is not always reproducible.

The results obtained with different compounds as eluents of pyruvate kinase are summarized in table 1. With ATP, the negative allosteric effector [14–16] at a concentration of 0.5 mM, the enzyme came off the column with a purification factor of 30. The specificity of the effectors as eluents is shown by the fact that other salts such as Tris-maleate and pyrophosphate, removed the enzyme from the column but gave preparations of much lower specific activity (table 1). The minimum concentration of Tris-maleate which eluted the enzyme was found to be 30 mM and the prepara-

Table 1
Elution of pyruvate kinase from CM-cellulose columns with different compounds. Experimental conditions as described in fig. 1. All eluents were dissolved in the equilibrating buffer. Numbers represent each separate experiment and the letters (a,b) two different eluents used in succession. The amount of protein poured over each column was between 10 to 20 mg, except in the last experiment in which it was only 1 mg.

Eluent	Concentration (mM)	Specific activity		Recovery (%)	Purification factor
		Initial	Final		
1. Tris-maleate	30*	1.3	7.3	80	5.5
2. Pyrophosphate (Na)	4.5*	1.3	7.6	75	5.9
3. Phosphate (Na)	14*	1.1	17.6	63	17.0
4a. α-Glycerophosphate	5	1.0	–	0	–
4b. Tris-maleate	50	1.0	4.4	50	4.4
5. FDP	0.3	1.2	42.6	63	35.5
6. ATP	0.5	1.2	36.3	55	30.0
7a. ADP	1.0	1.4	–	0	–
7b. FDP	1.0	1.4	47.6	52	34.0
8a. Fructose 6-phosphate	1.0	1.4	–	0	–
8b. FDP	1.0	1.4	29.5	55	21.0
9a. AMP	1.0	1.2	–	0	–
9b. FDP	1.0	1.2	22.3	80	18.5
10. UTP	1.0	1.4	17.6	64	12.5
11. FDP	0.5	4.4	167	84	38.0

* Minimal concentration necessary for elution.

tion was only 5-fold purified. A linear gradient of inorganic phosphate also removed the enzyme but a higher concentration was required (up to 14 mM), and the purification factor obtained was lower than that found by elution with FDP or ATP alone.

It has been found that UTP moderately inhibits pyruvate kinase activity [20]. This nucleotide also eluted the enzyme but it is less efficient than ATP. Other metabolites, such as fructose 6-phosphate, AMP, ADP and α-glycerophosphate, used as eluents at a concentration of 1 mM, did not remove the enzyme from the column. By further addition of 1 mM FDP the activity was partially recovered but the specific activity was somewhat variable. The pretreatment of the column with any eluent apparently lessens the efficiency of FDP elution. The results of table 1 also show that α-glycerophosphate, at a concentration of 5 mM, was unable to elute the enzyme which was partially removed with 50 mM of Tris-maleate.

It should be mentioned that although the results with the same batches of enzyme and exchanger were reproducible, there was a certain variability from one experiment to another especially in the extent of the purification. In this connection is must be added that when phosphoenolpyruvate, one of the substrates of the enzyme, was used as eluent the behavior of the column was quite variable. With few exceptions, at a concentration of 1 mM of this compound the enzyme remained in the column but was eluted at 3 mM, with a recovery of about 50% and the peak fractions were 30-fold purified (results not shown in the table).

The specific activity of the starting material in most of the experiments was about 1.3 units per mg, when a more purified preparation was used (DEAE-cellulose fraction [16]), the specific activity of the peak fractions increased to 167, which represented a purification of about 1100-fold over the crude extract.

In addition, other experiments performed with a mixture of both types of liver pyruvate kinase showed that the elution with FDP provides another procedure for the separation of both isoenzymes. Whereas the L type is readily eluted with the effector the other form (M type) is not, but can be removed from the column with 120 mM of Tris-maleate. A separate experiment with the pyruvate kinase from heart muscle confirms the above result, that is, the activity of this M type enzyme is not selectively eluted by FDP.

The procedure described in this communication has been applied to the preparation of pyruvate kinase free from fructose diphosphatase which interferes in the study of the FDP effect on the former enzyme. This could not be achieved previously because of the similar chromatography properties of both enzymes. Pyruvate kinase and fructose diphosphatase eluted from a CM-cellulose column at the same position when linear gradients of buffer were

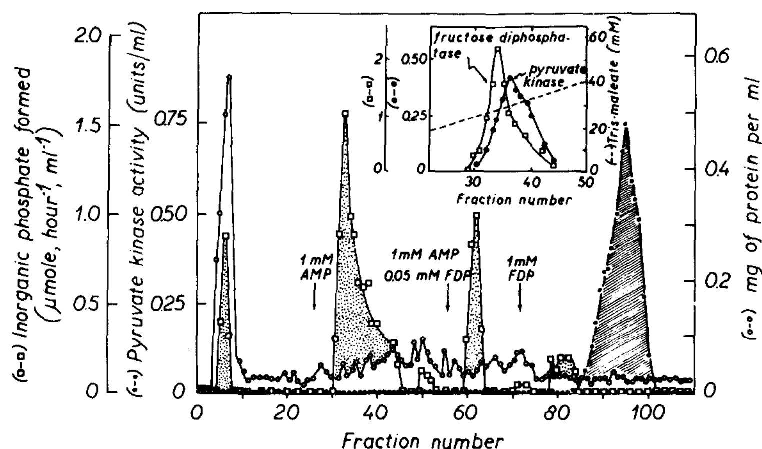

Fig. 2. Separation of pyruvate kinase and fructose diphosphatase by a preferential effector elution. Experimental conditions as in fig. 1. The starting material contained 10 mg of protein. After washing the column with 50 ml of the equilibrating buffer, different eluents, shown by the arrows, were applied. Inset: elution of the enzymes with a linear gradient of Tris-maleate buffer, pH 6.0. Enzyme activities are expressed as in the main figure.

applied (see the inset of fig. 2). When pyrophosphate was used instead of Tris-maleate a similar result was obtained. Furthermore both activities were not separated by other classical fractionation procedures.

The elution profile in fig. 2 shows that 1 mM AMP removed a considerable amount of fructose diphosphatase (about 70%) resulting in a 54-fold increase in its specific activity. Further addition of a mixture of 0.05 mM FDP and 1 mM AMP removed most of the remaining enzyme. This treatment did not elute the pyruvate kinase, which was finally recovered by a selective elution with 1 mM FDP.

By means of this simple and rapid method of effector elution, a preparation of pyruvate kinase completely free of contaminating fructose diphosphatase can be obtained in a few hours. This gives a practical example of the usefulness of the chromatographic procedure described in this paper.

From the results presented here it is possible to expect that when a regulatory enzyme is very loosely adsorbed to an ion exchanger, it can be preferentially eluted by its allosteric effectors, substrates or related compounds.

Acknowledgements

We are grateful to Dr. Luis F.Leloir for helpful discussions and criticism. We also wish to express our appreciation to Dr. Susana Passeron and Dr. R.Epand for their help in the English version of the manuscript and to other members of the Instituto de Investigaciones Bioquimicas for their interest and constructive comments.

Supported in part by research grants from the U.S. Public Health Service (No. GM 03442) and the Consejo Nacional de Investigaciones Cientificas y Técnicas (R.Argentina). H.C. and L.J.de A. are career investigators of the latter institution.

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