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The effect of cations on the electrophoretic mobility and substrate binding properties of pyruvate kinase*

A number of enzyme systems are stimulated by, or show an absolute requirement for, a univalent cation[1]. A theory of enzyme activation by univalent cations which satisfies available evidence is to consider such enzymes to be flexible and their shape dependent on the cationic environment. Thus, when activator ions are bound to charged groups on the protein, the conformation is assumed to be such as to expose the active site which would remain buried in the conformation resulting from the binding of non-activator ions[1]. Positive evidence of a conformational change between such an enzyme in a medium containing K^+, an activator, and one containing Li^+, a non-activator, would provide support for the above theory. Various attempts have been made to show a differential effect of various univalent cations on some physical parameter of a univalent cation-activated enzyme[2-5]. The present study reports the electrophoretic mobility of pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) in various environments. In addition, substrate binding studies have been made to supplement the conclusions reached by electrophoretic studies.

In using electrophoretic and gel filtration techniques the time required for equilibrium to be reached between enzyme, activators and substrate is negligible compared with the period of electrophoresis and chromatography and it is assumed that at near saturating concentrations of activators and substrate the protein exists as a single complex with no free protein.

Electrophoretic mobility was measured using a Perkin–Elmer Model 38 electrophoresis unit. Rabbit muscle pyruvate kinase (Calif. Biochem. Co. A grade) was suspended in the desired buffer medium and equilibrated with the same medium on Bio-gel P-2 columns (Bio-Rad Laboratories). The monocyclohexylammonium salt of phosphoenolpyruvate and Tris–ADP were adjusted in solution to pH 7.4 with Tris. The buffering system was 0.01 M Tris–HCl (pH 7.4) in all experiments and the pH remained within $\pm$ 0.05 pH units.

Table I shows the results of experiments in which the electrophoretic mobility

TABLE I

THE EFFECT OF THE CATIONIC ENVIRONMENT ON THE ELECTROPHORETIC MOBILITY OF PYRUVATE KINASE

Each electrophoresis medium contains 0.01 M Tris–HCl (pH 7.4) and the concentration of salts indicated. The protein concentration was 2 mg/ml, the temperature 0° and the power 2 W. Data reported are the means of at least two replicate determinations.

Expt. No.	*Additions to electrophoresis medium*	*Resistance of medium (Ω)*	*Current (A)*	*Mobility × 10^5 ($cm^2 \cdot V^{-1} \cdot sec^{-1}$)*
1	0.1 M KCl	129	0.016	1.30 $\pm$ 0.03
2	0.1 M LiCl	174	0.014	1.32 $\pm$ 0.02
3	0.1 M KCl, 0.01 M $MgCl_2$	109	0.018	0.99 $\pm$ 0.02
4	0.1 M LiCl, 0.01 M $MgCl_2$	145	0.015	0.81 $\pm$ 0.03

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of pyruvate kinase was measured in media containing the chloride salts of various cations. No appreciable difference was detected between the mobility in 0.1 M KCl *versus* that in 0.1 M LiCl, both cases yielding an anodic migration of approx. $1.3 \cdot 10^{-5}$ $cm^2 \cdot V^{-1} \cdot sec^{-1}$. Thus, any conformational variations in pyruvate kinase caused by an exchange of non-activating Li^+ by activating K^+ cannot be detected by the technique as applied here.

When 0.01 M $MgCl_2$ (Table I, lines 3 and 4) was added to the enzyme in both the K^+ and Li^+ environment, a differential decrease in mobility occurred so that the enzyme in K^+ *plus* Mg^{2+} migrated at $9.9 \cdot 10^{-6}$ $cm^2 \cdot V^{-1} \cdot sec^{-1}$ whereas the mobility of the enzyme in Li^+ *plus* Mg^{2+} was $8.1 \cdot 10^{-6}$ $cm^2 \cdot V^{-1} \cdot sec^{-1}$. The above results could indicate either a conformational difference or an excess of Mg^{2+} binding to the protein in the Li^+ environment. Meaningful measurements of metal ion binding to the enzyme by techniques equivalent to equilibrium dialysis were not possible in media where the concentrations of such ions were massive in comparison to the molar concentration of enzyme. That Mg^{2+} can affect the fine structure of pyruvate kinase was clearly demonstrated by the ultraviolet difference spectrophotometric studies of KAYNE AND SUELTER[6]. The work of MELCHIOR[7] indicates, however, that Mg^{2+} functions in the pyruvate kinase reaction by forming a Mg–ADP complex.

There is evidence to suggest that the binding of substrates may cause a conformational change in pyruvate kinase[6]. It was of interest therefore to determine whether electrophoresis could detect an effect of K^+ *versus* Li^+ on the enzyme in the presence of substrates. The electrophoretic mobility of pyruvate kinase was measured therefore in the presence of either phosphoenolpyruvate or ADP at concentrations greater than 90% of that required for maximal activity[8]. During preliminary investigations of electrophoresis in the presence of phosphoenolpyruvate a progressive decrease in electrophoretic mobility was observed, whether K^+ or Li^+ was the predominant univalent cation in the medium. If, after equilibrating with the desired medium, the enzyme was incubated at 4° for 1.5 h prior to electrophoresis, a constant mobility was always obtained. No further investigation of the effect of phosphoenolpyruvate on the initial changes in mobility of the enzyme has been conducted.

Table II shows a comparison of the effect of substrates on the electrophoretic

TABLE II

THE INFLUENCE OF CATIONIC ENVIRONMENTS ON THE ELECTROPHORETIC MOBILITY OF PYRUVATE KINASE IN THE PRESENCE OF SUBSTRATES

The electrophoretic conditions were as in Table I except for the additions as indicated. KCl, LiCl, 0.1 M; $MgCl_2$, 0.01 M; phosphoenolpyruvate, $1.28 \cdot 10^{-4}$ M; ADP, $7.3 \cdot 10^{-4}$ M. Data reported are the means of at least two replicate determinations.

Expt. No.	*Additions to electrophoresis medium*	*Resistance of medium (Ω)*	*Current (A)*	*Mobility × 10^5 ($cm^2 V^{-1} sec^{-1}$)*
1	KCl, $MgCl_2$, phosphoenolpyruvate*	107	0.018	0.97 ± 0.03
2	LiCl, $MgCl_2$, phosphoenolpyruvate*	143	0.016	0.82 ± 0.03
3	KCl, $MgCl_2$, ADP	108	0.018	1.19 ± 0.03
4	LiCl, $MgCl_2$, ADP	144	0.016	0.97 ± 0.02

* The protein was incubated in the electrophoresis medium for 1.5 h prior to the electrophoresis.

mobility of pyruvate kinase in either a medium containing K^+ and Mg^{2+} or one containing Li^+ and Mg^{2+}. Results in the presence of phosphoenolpyruvate after a period of incubation of 1.5 h at 4° (see Table II, Experiments 1 and 2) were similar to those shown in Table I (Expts. 3 and 4) for the mobility of the enzyme in the same cationic environment but in the absence of phosphoenolpyruvate. When phosphoenolpyruvate was replaced by ADP (Table II, Expts. 3 and 4) the electrophoretic mobility was increased no matter whether K^+ or Li^+ was the predominant univalent cation species present. Thus, the influence of a given substrate on the electrophoretic mobility is the same no matter whether K^+ or Li^+ is present.

Substrate binding was studied using the procedure of HUMMEL AND DREYER[9] in which the binding of a small molecular weight species to a protein takes place during the passage of the protein sample through a gel filtration column. In those experiments in which ADP binding to pyruvate kinase was studied, the concentrations of both enzyme and ADP were calculated by developing simultaneous equations for their absorbance at 280 and 260 mμ and solving for ADP or protein concentration. Figs. 1A

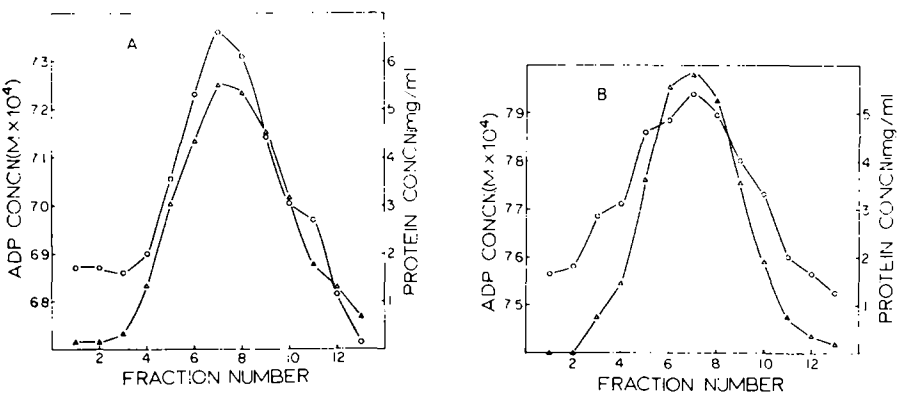

Fig. 1. The binding of ADP to pyruvate kinase as influenced by univalent cation salts. Samples containing 6 mg of pyruvate kinase in 0.5 ml of medium were chromatographed on Bio-gel P-2 columns, 11 cm × 1.1 cm, equilibrated with the same medium. The flow rate was 0.3 ml/min. The medium contains (A) 0.01 M Tris·HCl (pH 7.4), 0.01 M $MgCl_2$, 0.1 M KCl, $6.87 \cdot 10^{-4}$ M ADP; (B) Tris and $MgCl_2$ as in A, 0.1 M LiCl, $7.57 \cdot 10^{-4}$ M ADP. The protein eluate was collected as samples of 3 drops from which were taken 0.1 ml aliquots for dilution for absorbance measurements. ○, ADP; △, protein.

and 1B present the results of experiments designed to determine the effect of K^+ *versus* Li^+ on the binding of ADP to pyruvate kinase. Calculations reveal that 1.7 molecules of ADP are associated with each molecule of enzyme no matter whether K^+ or Li^+ is present in the equilibrating medium.

Phosphoenolpyruvate binding was studied by a similar procedure and calculation showed that approximately two excess molecules of phosphoenolpyruvate are associated with each molecule of enzyme no matter whether K^+, an activating cation, or Li^+, a nonactivating cation, are predominant in the protein environment. Phosphoenolpyruvate binding to pyruvate kinase in an activator environment has been previously reported by REYNARD *et al.*[8].

The results indicate that one cannot envisage K^+ causing a conformational

change in the enzyme thereby exposing buried active sites. For it is obvious that sites for substrate binding are available whether an activator univalent cation is present or not. Thus, a proposed enzyme conformation[5] induced by K^+ and essential for phosphoryl transfer, could be attained either before or after substrate binding but must be important at some later stage in the overall mechanism. One can imagine that any conformational change involved need be only small, perhaps explaining why so little success has been obtained from most of the existing methods of detecting conformational variations.

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Department of Botany, Oregon State University GRAHAM F. BETTS*
Corvallis, Oreg. (U.S.A.) HAROLD J. EVANS

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* Present address: School of Biological Sciences, University of Sussex, Brighton BN1 9QG, England.

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The inhibition of univalent cation activated enzymes by tris(hydroxymethyl) aminomethane

Many enzyme systems are stimulated by or show an absolute requirement for a univalent cation[1]. K^+ is commonly the most effective ion, and in general, enzymes requiring univalent cations are not activated by Li^+. Various organic cations such as $Tris^+$ and $tetramethylammonium^+$ also fail to activate and have been used widely, therefore, in buffering systems where univalent cation requirements are being studied. In addition to failing to substitute for K^+ as a univalent cation activator, Li^+ frequently has been shown to inhibit certain enzymes in environments containing K^+ (refs. 2–6). The type of inhibition has rarely been studied adequately, but occasionally, has been shown to be competitive with K^+ (refs. 2, 6). The question then arises whether non-activators of enzymes such as Tris can also inhibit competitively, in which case