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# PURIFICATION AND MOLECULAR PROPERTIES OF THE AMP-ACTIVATED PYRUVATE KINASE FROM *ESCHERICHIA COLI*

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## Summary

The AMP-activated pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) from *Escherichia coli* has been purified 200 times through a three-step procedure which gives a homogeneous preparation with a specific activity of 110.

The enzyme appears to be a tetramer of molecular weight 190 000. Subunits (molecular weight 51 000) show a single amino-terminal amino acid (serine) and appear as a single band in polyacrylamide gel electrophoresis in sodium dodecyl sulphate.

The enzyme crystallizes in conditions of reduced dielectric constant of the solvent in the pH range 6.5—7.5.

Kinetic and regulatory properties of the purified enzyme are similar to those described for crude preparations of the enzyme.

## Introduction

Two forms of pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) have been described in *Escherichia coli* [1]. They differ in their response to allosteric effectors [1—4] and undergo opposite changes in their intracellular levels, according to growth conditions of the cells [2,5,6]. These two forms do not appear to be conformers of the same protein, since they are not easily interconvertible [1,2]. Their presence within the cell can be demonstrated by in situ studies [5].

Different criteria have been suggested for their nomenclature [1,5]. The fructose diphosphate-activated form (Pk-F), on the basis of its chromatographic behaviour on DEAE-cellulose, has also been called type I pyruvate kinase (Pk-I). The form activated by AMP (Pk-A) and by glucose 6-phosphate, ribose 5-phosphate and other sugar phosphates has also been indicated as type II (Pk-II).

A detailed study of the two forms of pyruvate kinase from *E. coli* would be

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interesting from a physiological and regulartory point of view, because the two forms appear to coexist, although in different amounts, in most metabolic conditions and consequently the total pyruvate kinase activity of the cell results from the contribution of both forms of enzyme. Moreover, from a molecular point of view, the understanding of the relationship between the two forms might throw light upon the mechanism underlaying the existence of multiple enzyme forms at a different control point of the glycolytic pathway in *E. coli*, namely at the level of phosphofructokinase [6,7].

While type I pyruvate kinase has been completely purified [2,3], attempts to purify type II have failed [4] because of instability of the enzyme. Taking advantage of the observation that the removal of KCl from partially purified preparations of type II pyruvate kinase resulted in rapid loss of activity, we have purified this form of enzyme in conditions of high ionic strength and undertaken its molecular characterization.

## Materials

Phospho*enol*pyruvate tricyclohexylammonium salt, ribose 5-phosphate, glucose 6-phosphate, ATP, ADP and AMP were purchased from Sigma (St. Louis, Mo., U.S.A.). Lactate dehydrogenase, NADH and standard proteins for molecular weight estimation were purchased from Boehringer (Mannheim, Germany). Acrylamide, methylenebisacrylamide, *N,N,N',N'*,-tetramethylenediamine and 2-mercaptoethanol were obtained from Eastman Kodak Co. (Rochester, U.S.A.); sodium dodecyl sulphate was from Serva Feinbiochemia (Heidelberg, Germany). Dextran Blue 2000 was purchased from Pharmacia (Uppsala, Sweden). Nutrient agar and nutrient broth were purchased from Oxoid (London, England). All other chemicals of reagent grade were produced by Merck (Darmstadt, Germany) or by C. Erba (Milano, Italy).

DEAE-cellulose (DE 32) and phosphocellulose (P 11) were purchased from Whatman Biochemicals, Ltd. (Springfield Mill, England).

Glass distilled water was used for preparation of all solutions.

## Methods

*Protein and chloride estimation.* Proteins were estimated according to a modification of the method of Lowry et al. [8]. Chlorides were assayed as described by Vogel [9].

*Polyacrylamide gel electrophoresis.* Routine polyacrylamide gel electrophoresis was performed according to the method of Ornstein [10] and Davis [11] on 6.5 × 6.5-cm slabs in an Uniscil apparatus. Samples containing 15—40 μg protein were applied to each well. The pherogram was developed with a current of 40 mA. Gels were stained with 1% Amido Black in acetic acid and destained in 7% acetic acid. In order to check the homogeneity of the purified enzyme preparation, electrophoresis was also performed at different polyacrylamide concentrations (7, 8, 9 and 10%) on gels prepared according to the method Hedrick and Smith [12] without upper gel, from a stock solution of 40% acrylamide.

Subunit molecular weight estimation by polyacrylamide gel electrophoresis in sodium dodecyl sulphate was performed according to Shapiro et al. [13], except that cytochrome *c* was used as internal standard.

*Molecular weight estimation.* Molecular weights of native proteins were estimated by gel filtration according to Andrews [14] on a 1.77 × 96 cm Sephadex G-200 fine column, equilibrated with 10 mM Tris, 1 mM EDTA, 2 mM mercaptoethanol, 100 mM KCl, pH 7.5. Void volume was measured as the elution volume of a Dextran Blue 2000 solution. Because of interactions between Dextran Blue and pyruvate kinase, the latter was never run in the presence of the former, but void volume was always measured before and after runs where pyruvate kinase was present and average values (if necessary) were taken for subsequent calculations.

*Amino-terminal analysis.* The amino-terminal amino acid was identified as dansyl derivative, according to Gray [15].

*Amino acid analysis.* Amino acid analysis was performed on a Beckman automatic amino acid analyzer, model 120 B, equipped with high sensitivity cuvettes. The separation of amino acids was obtained on a single column (0.9 × 50 cm) filled with M70 Beckman resin, according to Spackman et al. [16] modified by Dévényi [17].

Cysteine was determined as cysteic acid according to the method of Spencer and Wold [18].

*Crystallization.* The procedure used for crystallization was adapted from that of Zeppezauer et al. [19]: plexiglass microcrystallizers were filled with 0.05 ml of enzyme solution (approx. 10 mg/ml), covered with dialysis membranes and allowed to equilibrate with different buffers in different conditions.

*Pyruvate kinase assay.* Pyruvate kinase activity was assayed by a coupled continuous spectrophotometric assay derived from the method of Bücher and Pfleiderer [20]. The standard assay mixture contained in a final volume of 1 ml: 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.5; 10 mM $MgCl_2$; 50 mM KCl; 14.4 units of crystalline lactate dehydrogenase; 0.12 mM NADH; 10 mM phospho*enol*pyruvate; 2 mM ADP and pyruvate kinase. The reaction was carried out at 25°C and the decrease in absorbance at 340 nm was recorded in a recording spectrophotometer.

One unit of enzyme is the amount of pyruvate kinase which catalyzes the formation of 1 μmol of pyruvate per min in the above conditions.

*Kinetic constants.* Kinetic constants were calculated according to Wieker et al. [21].

*Strain and culture conditions. E. coli* K 12 J 53 [22], an auxotroph for methione and proline, has been used throughout this work. The strain was maintained on nutrient agar. Cultures were grown in the synthetic medium of Ashworth and Kornberg [23], with the addition of methionine, proline (40 μg/ml each) and nutrient broth (0.5%, v/v); 25 mM glucose was used as carbon source. Sterile medium (10 l) was inoculated with 200 ml of a liquid culture (approx. 1 mg dry weight of cells/ml) and the culture was grown aerobically at 37°C in a New Brunswick Microferm fermentor under constant mixing (500 rev./min) and with an air supply of 5 l/min.

Growth was interrupted in the late logarithmic phase at a cell density of 1.0—1.2 mg dry weight/ml. The density of the culture was measured turbidime-

trically at 680 nm; absorbance readings were converted into cell density (in mg dry weight/ml) by multiplying by a factor of 0.68. Bacterial cells were harvested by centrifugation at 9000 × *g* for 8 min, the precipitate was washed twice with 5 mM phosphate buffer, 1 mM EDTA, 2 mM mercaptoethanol, pH 7.5, and finally resuspended in the same buffer at a cell concentration of approx. 100 mg dry weight/ml.

*Preparation of the crude extract.* The cells were broken by exposure in small aliquots for 6 min to the output of a 100 W MSE sonicator at a frequency of 20 000 Hz, with an amplitude of 8 μm, at 0° C.

The crude extract was spun at 46 000 × *g* for 40 min in order to remove unbroken cells and membrane fragments. The supernatant was stored at −60° C.

*Preparation of the fructose diphosphate-activated pyruvate kinase.* The fructose diphosphate-activated pyruvate kinase (type I) used in this work was prepared as previously described [2] from the same bacterial strain (*E. coli* K 12 J 53).

## Results

*Purification of the AMP-activated pyruvate kinase.* Step 1: DEAE-cellulose chromatography. 344 ml of crude extract obtained from approx. 35 g (dry weight) of bacterial cells have been applied to a 6 × 35 cm DEAE-cellulose column equilibrated with 5 mM phosphate buffer, 1 mM EDTA, 2 mM mercaptoethanol, pH 7.5. Elution was performed first by washing with approx. 2 l of the equilibrating buffer and subsequently by applying a linear gradient of KCl from 0 to 0.5 M in the same buffer, for a total volume of 1800 ml. The flow rate was 70 ml/h. The elution profile of the portion of the chromatogram containing pyruvate kinase is shown in Fig. 1: pyruvate kinase activity appears in two peaks; the AMP-activated form of the enzyme is present in the second peak, eluted at 0.17—0.20 M KCl.

Fractions from this peak containing more than 7 units/ml were pooled; the electrophoretic pattern of proteins in this pool is shown in Fig. 4a.

The pool (160 ml) was dialyzed against 3 × 2 l of 10 mM Tris, 1 mM EDTA, 2 mM mercaptoethanol, 150 mM KCl, pH 7.5, and then concentrated to a small volume (7 ml) by ultrafiltration under $N_2$ pressure in an Amicon ultrafiltration cell equipped with a PM-30 membrane. The precipitate formed during concentration was removed by centrifugation at 46 000 × *g* for 30 min.

Step 2: affinity chromatography on phosphocellulose. This resin has been selected for two sets of reasons: on one hand, observations made on the fructose diphosphate-activated pyruvate kinase from *E. coli* [2] showed that this form of enzyme could bind to the resin at pH 7.5 and at low ionic strength and could be specifically eluted by the allosteric effector of the enzyme (fructose 1,6-diphosphate); on the other, the resin contains glucose phosphate groups, which could mimick one of the effectors of type II pyruvate kinase (namely glucose 6-phosphate) or could supply phosphate groups for other phosphate- or phosphoryl-binding sites on the enzyme.

Preliminary observations indicated that indeed the AMP-activated form of the enzyme did bind to the resin and that such a binding, in the pH range from

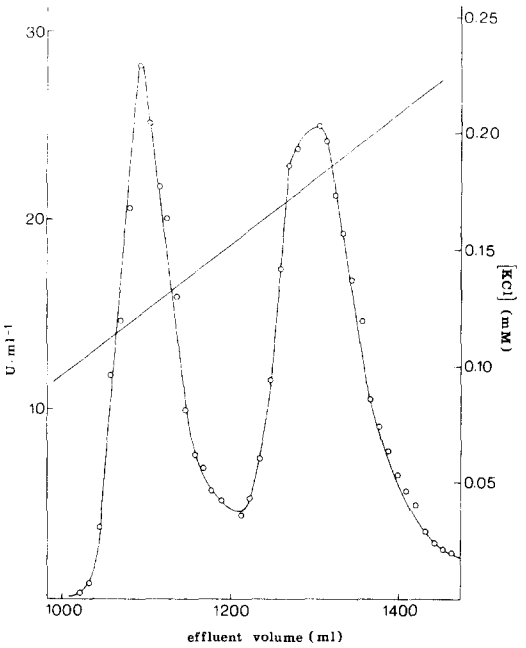


**Fig. 1. Elution pattern of pyruvate kinase activity from DEAE-cellulose. ○, pyruvate kinase activity; ———, KCl concentration.**

7.5 to 8.5, required relatively low ionic strength. The conditions finally adopted are a compromise between the need for high salt concentration for stability of the enzyme (see below) and the need of low ionic strength for binding to the resin: a 2.5 × 65 cm column was packed with phosphocellulose equilibrated in 10 mM Tris, 1 mM EDTA, 2 mM mercaptoethanol, pH 7.5; the enzyme was applied to the column in a small volume (7 ml) of a KCl-containing buffer.

The binding of the enzyme to phosphocellulose is not rapidly reversed by the presence of KCl in the eluting buffer; the column was therefore washed with approx. 1250 ml of the equilibrating buffer containing 150 mM KCl and 1 mM fructose 1,6-diphosphate. The latter compound was added in order to remove from the resin any contamination by type I pyruvate kinase [2].

Elution was completed by running through the column 600 ml of 100 mM phosphate buffer, 1 mM EDTA, 2 mM mercaptoethanol, 150 mM KCl, pH 8.0. The flow rate was 30 ml/h.

From the chromatogram reproduced in Fig. 2, it appears that most proteins are eluted from the column in less than two void volumes, while a smaller peak appears in the effluent at 4—5 void volumes. Type II pyruvate kinase is eluted in a very sharp peak by phosphates. In some preparations, a different behaviour was observed, that is, type II pyruvate kinase was eluted together with the "late" protein peak, in the absence of phosphates.

Fractions with specific activity (units/$A_{280\ nm}$) higher than 20 were pooled: the electrophoretic pattern of proteins of this pool is shown in Fig. 4b; the purification factor over the previous step was 35. The pool (25 ml) was

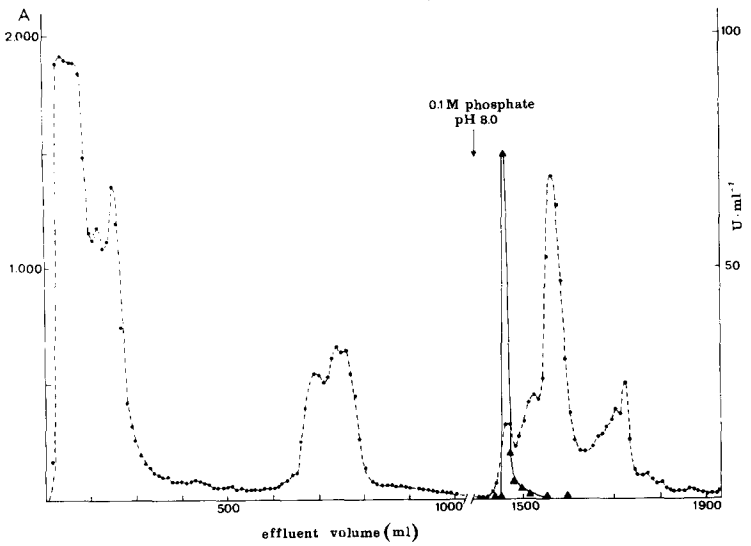


Fig. 2. Elution profile of protein (●) and of pyruvate kinase activity (▲) from phosphocellulose.

dialyzed against 2 × 1 l of 10 mM Tris, 1 mM EDTA, 2 mM mercaptoethanol, 100 mM KCl, pH 8.5.

Step 3: DEAE-Sephadex chromatography. The enzyme preparation was applied to a DEAE-Sephadex column (1.8 × 90 cm) equilibrated with 10 mM Tris, 1 mM EDTA, 2 mM mercaptoethanol, 100 mM KCl, pH 8.5. Elution was performed first with 200 ml of the equilibrating buffer and subsequently with a convex gradient of KCl (875 ml) from 0.1 to 0.5 M, whose shape is reproduced in Fig. 3, together with the elution profiles of pyruvate kinase and of protein. Flow rate was 15 ml/h. Fractions showing, within experimental error,

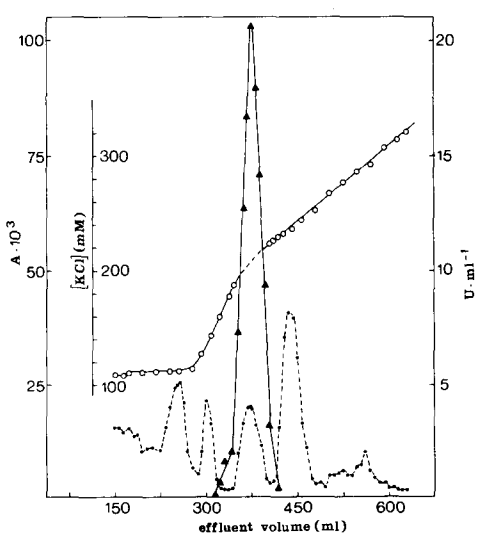


Fig. 3. Chromatography of the AMP-activated pyruvate kinase on DEAE-Sephadex. ●, absorbance at 280 nm; ▲, pyruvate kinase activity; ○, KCl concentration.

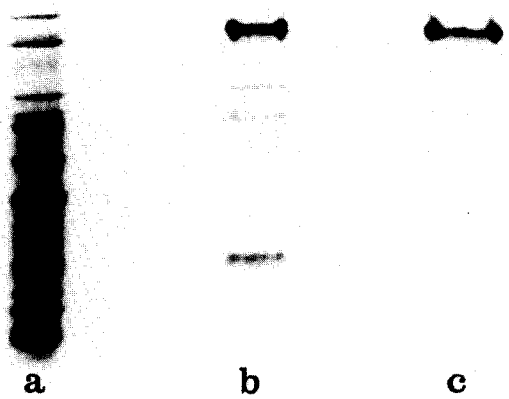


Fig.4. Polyacrylamide gel electrophoresis of type II pyruvate kinase from *E. coli* at different stages of purification. a, after DEAE-cellulose step; b, after phosphocellulose step; c, after DEAE-Sepahdex step.

constant specific activity (units/$A_{280\ nm}$) were pooled: this pool appears as a single band in polyacrylamide gel electrophoresis (Fig. 4c). The purification factor over the previous step was 1.8.

The results of purification are summarized in Table I.

*Homogeneity.* The purification procedure described here gives reproducibly an enzyme preparation, the homogenous nature of which is suggested by the convergence of several criteria: (1) amino acid analysis: different enzyme preparations gave consistent results upon amino acid analysis; (2) N-terminus analysis: only a single dansyl-amino acid was detectable after dansylation and hydrolysis of enzyme preparations; (3) polyacrylamide gel electrophoresis in sodium dodecyl sulphate: the enzyme gave a single band even when loaded on the gels in high amounts; (4) polyacrylamide gel electrophoresis at different polyacrylamide concentrations: a single band was detectable at all concentrations tested (Table II).

*Stability.* The enzyme was found to lose activity in the absence of mercap-

TABLE I

PURIFICATION OF THE AMP-ACTIVATED FORM OF PYRUVATE KINASE FROM *E. Coli*

| Purification step | Volume (ml) | Protein (mg/ml) | Activity (units/ml) | Total units | Specific activity (units/mg) | Purification * | Yield |
|---|---|---|---|---|---|---|---|
| Crude extract | 344 | 48.50 | 25.8 * | 8875 * | 0.53 * | 0 | 100 * |
| DEAE-cellulose + dialysis | 174 | 9.04 | 15.8 | 2749 | 1.75 | 3.3 | 31.0 |
| Phosphocellulose + dialysis | 29 | 0.51 | 31.34 | 909 | 61.50 | 116 | 10.2 |
| DEAE-sephadex | 56 | 0.133 | 14.76 | 826 | 110 | 208 | 9.3 |

* Both forms of pyruvate kinase contribute to the activity of crude extracts. Bacterial cells grown in the conditions of this paper contain approximately equal amounts of the two forms.

TABLE II

ELECTROPHORETIC MOBILITY OF THE AMP-ACTIVATED FORM OF PYRUVATE KINASE AT DIFFERENT POLYACRYLAMIDE CONCENTRATIONS

| Polyacrylamide concentration (%) | Mobility * |
|---|---|
| 7 | 0.311 |
| 8 | 0.280 |
| 9 | 0.217 |
| 10 | 0.182 |

* Relative to Bromophenol Blue.

toethanol and KCl; the highest stability was obtained in the presence of 2 mM mercaptoethanol and of 150 mM KCl. The enzyme, dissolved in 10 mM Tris, 1 mM EDTA, 2 mM mercaptoethanol, 150 mM KCl, pH 8.0, at concentrations ranging from 0.5 to 10 mg/ml did not appreciably lose activity when stored at 4° C for up to 6 months.

The stabilizing efficiency of $K^+$, $Na^+$ and $NH_4^+$ (150 mM), tested on diluted solutions of the enzyme (6.4 μg protein/ml, in 10 mM Tris, 1 mM EDTA, 2 mM mercaptoethanol, pH 7.5) resulted in the following order: $K^+ > NH_4^+ > Na^+$.

*Molecular weight.* The molecular weight of the native protein, estimated by gel filtration, gave a value of 190 000 ± 10 000. This value is in good agreement with the data published by Waygood et al. [4] and falls well within the range of molecular weights observed for other pyruvate kinases [24].

Electrophoresis of pyruvate kinase II in polyacrylamide gel, containing sodium dodecyl sulphate gave a single band corresponding to a molecular weight of 51 000 ± 5%. Like all other pyruvate kinases described so far, the AMP-activated pyruvate kinase from *E. coli* appears to be a tetrameric protein. The fact that a single band is detected in polyacrylamide gel electrophoresis in sodium dodecyl sulphate suggests that only one type of subunit is present: this suggestion is strengthened by the results on the $NH_2$ terminus analysis (see below).

*Amino acid analysis.* The results of the amino acid analysis of type II pyruvate kinase are summarized in Table III: the total number of amino acid residues per subunit molecular weight of 51 000 is 482. A low content in aromatic amino acids, such as the one reported here, has been observed in the fructose diphosphate-activated pyruvate kinase from *E. coli* [3] as well as in the pyruvate kinase from rabbit muscle [25].

*Amino terminus analysis.* A single amino acid, serine, has been detected as amino terminus in the AMP-activated pyruvate kinase from *E. coli* (Fig. 5). This observation differs from published data for the fructose diphosphate-activated pyruvate kinase from *E. coli* [3] and for the enzymes from rabbit muscle [25], human erythrocytes [26] and yeast [24].

*Crystallization.* Crystallization of the AMP-activated form of pyruvate kinase can be obtained from a solution in 10 mM Tris, 1 mM EDTA, 2 mM mercaptoethanol, 150 mM KCl, only in the presence of agents reducing the dielectric constant of the solvent, such as 2-methyl-2,4-pentanediol [27], within a relatively narrow range of pH (6.5—7.5). The crystals obtained in these conditions

TABLE III

AMINO ACID COMPOSITION OF PYRUVATE KINASE II

| Amino acid | Residue per subunit of molecular weight 51 000 | | | Average (extrapolated) | Integer No. |
|---|---|---|---|---|---|
| | 24 h | 48 h | 72 h | | |
| Lys | 28.39 | 28.56 | 29.15 | 28.7 | 29 |
| His | 6.69 | 7.29 | 6.79 | 6.92 | 7 |
| Arg | 30.07 | 29.78 | 30.96 | 30.27 | 30 |
| Asp | 55.78 | 54.65 | 56.38 | 55.60 | 56 |
| Thr [a] | 29.18 | 27.88 | 27.80 | 29.88 | 30 |
| Ser [a] | 25.90 | 22.45 | 21.36 | 28.53 | 28 |
| Glu | 37.62 | 37.83 | 37.57 | 37.67 | 38 |
| Pro | 13.78 | 14.72 | 14.16 | 14.22 | 14 |
| Gly | 43.29 | 44.75 | 43.23 | 43.76 | 44 |
| Ala | 53.93 | 53.78 | 54.41 | 54.04 | 54 |
| Val [b] | 42.53 | 44.65 | 46.55 | 46.55 | 47 |
| Met | 15.12 | 13.40 | 15.68 | 14.73 | 15 |
| Ile [b] | 24.90 | 24.94 | 26.00 | 26.00 | 26 |
| Leu | 43.38 | 42.67 | 42.66 | 42.90 | 43 |
| Tyr | 5.67 | 5.50 | 5.88 | 5.68 | 6 |
| Phe | 9.07 | 8.94 | 9.19 | 9.06 | 9 |
| Cys [c] | 6.00 | — | — | — | 6 |
| Trp [d] | | | | | |

Total number of amino acids = 482

[a] Extrapolated to zero time.
[b] Maximum values taken.
[c] Determined as cysteic acid.
[d] Not assayed.

are reproduced in Fig. 6: the average dimensions were $120 \times 30 \times 30\ \mu m^3$.

*Kinetic and regulatory properties.* Kinetic and regulatory properties of this form of enzyme have been studied by Waygood et al. [4] on partially purified preparations: on the whole, the regulatory properties of the purified enzyme confirm these previous data.

When phospho*enol*pyruvate is used as the variable substrate, at near saturating ADP concentrations (2 mM) and pH 7.5, only a limited cooperativity

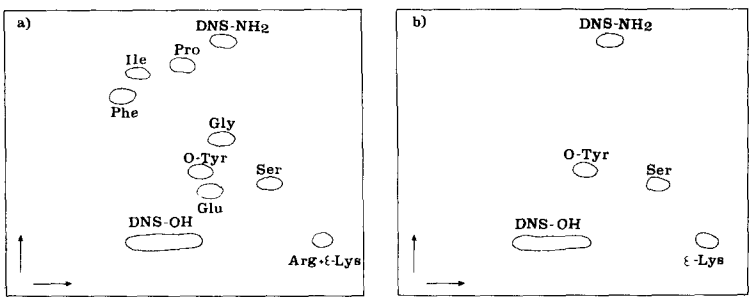


Fig. 5. Bidimensional chromatography of dansyl-amino acids. (a) Co-chromatography of an hydrolysate of dansylated pyruvate kinase and *N*-dansyl derivatives of arginine, serine, glutamic acid, glycine, phenylalanine, isoleucine, proline. (b) Chromatography of the hydrolysate of dansylated pyruvate kinase alone.

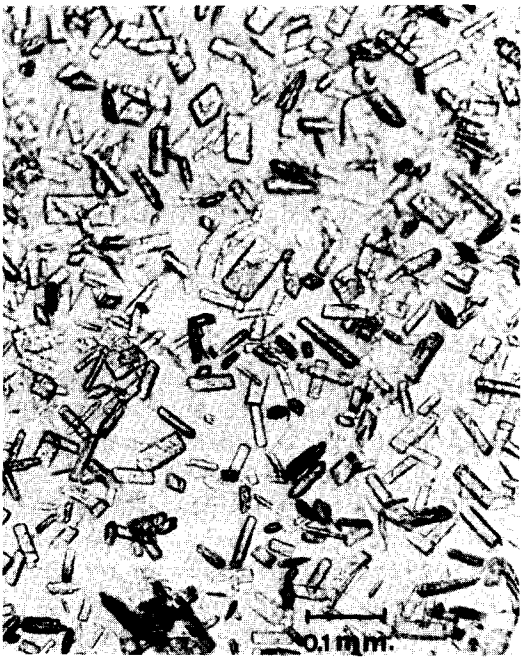


Fig. 6. Crystals of the AMP-activated pyruvate kinase from *E. coli*.

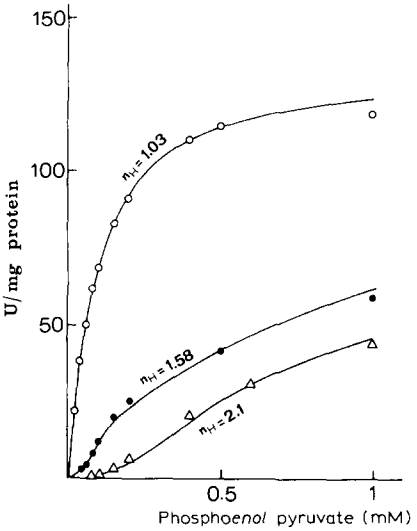


Fig. 7. Effect of inorganic phosphate on the activity of type II pyruvate kinase. Assay conditions as described under Methods. •, no additions; △, in the presence of 1 mM phosphate; ○, in the presence of 1 mM phosphate and 1 mM ribose 5-phosphate.

among phospho*enol*pyruvate-binding sites is observed (see Table IV). AMP (2 mM), glucose 6-phosphate (1 mM) and ribose 5-phosphate (1 mM) all activate the enzyme by increasing $V$ and decreasing $K_m$. Inorganic phosphate (1 mM) acts as negative effector decreasing $V$ and increasing $N_H$($N_H$ = 2.1); this inhibition is removed by the addition of ribose 5-phosphate (Fig. 7).

At saturating phospho*enol*pyruvate concentrations (5 mM), the enzyme displays normal Michaelis-Menten kinetics with respect to ADP with a $K_m$ of 0.17 mM, but sigmoidicity appears at non-saturating phospho*enol*pyruvate concentrations (0.5 mM), with a $K_m$ of 0.36 mM. Activation by ribose 5-phosphate is observed at both phospho*enol*pyruvate concentrations ($K_m$ = 0.12 mM). At

TABLE IV

KINETIC PARAMETERS FOR PHOSPHO*ENOL*PYRUVATE

Initial velocities were assayed as described under Methods at phospho*enol*pyruvate concentrations ranging from 20 mM to 50 μM.

| Addition | $K_m$ (mM) | V (units/mg protein) | $N_H$ |
|---|---|---|---|
| None | 0.82 | 109.9 | 1.58 |
| 2 mM AMP | 0.19 | 123.8 | 1.03 |
| 1 mM glucose 6-phosphate | 0.13 | 111.3 | 1.00 |
| 1 mM ribose 5-phosphate | 0.07 | 147.5 | 0.99 |

saturating phospho*enol*pyruvate concentration, inorganic phosphate acts as a non-competitive inhibitor with respect to ADP.

## Discussion

The three-step purification procedure described here yields a homogeneous and stable enzyme preparation, whose gross molecular properties fit well within the general architecture of pyruvate kinases from other sources (a tetrameric protein, of molecular weight of approx. 200 000, with most likely identical subunits, poor in aromatic amino acids).

If one takes into account the fact that in crude extracts from cells grown aerobically on glucose, which have been used as starting material for this purification, the two forms of pyruvate kinase are present in roughly equivalent amounts, the yield of the purification (approaching 20%) appears satisfactory.

The regulatory properties shown by the purified enzyme, as well as by crude preparations [4], are particularly interesting, especially if the variations in intracellular levels of enzyme in different growth conditions are taken into account. In fact the physiological significance of the strong activation by intermediates of the phosphogluconate pathway, suggested by Waygood et al. [4], could be accepted for growth in aerobic conditions, but appears unlikely in anaerobic conditions, when the AMP-activated pyruvate kinase is maximally depressed [6]. In these conditions the primary regulatory aspect would be connected with the response to AMP levels, as suggested by Kotlarz et al. [6].

One can drow a preliminary comparison between the main chemical and molecular properties of the two forms of pyruvate kinase from *E. coli*. Not only do they differ in molecular weight, but significant differences can be found between the amino acid composition of the AMP-activated pyruvate kinase (Table III) and the fructose diphosphate-activated form of the enzyme [3]: such differences concern lysine, arginine, glutamic acid and isoleucine. The finding of serine as the amino-terminal amino acid in the AMP-activated form is another important difference with respect to the fructose diphosphate-activated pyruvate kinase [3].

If also the kinetic and regulatory properties of the two forms are taken into account, as well as the regulation of their levels in different growth conditions [1,6], it seems reasonable to conclude that the two forms are different proteins.

Yet the fact that the presence of two forms of pyruvate kinase appears to be limited to *Enterobacteriaceae* (Kornberg, H.L. and Malcovati, M., unpublished) implies that their origin as separate proteins has been a relatively recent event (in evolutionary terms). It might then be interesting to further analyze the molecular and kinetic properties of these two forms in the attempt to understand the mechanism of their appearance, which could be connected with the presence in *E. coli* of enzyme duplications at the phosphofructokinase level [6,7].

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