Biochimica et Biophysica Acta, 570 (1979) 248—258

BBA 68825

TWO FORMS OF PYRUVATE KINASE IN *ESCHERICHIA COLI*

A COMPARISON OF CHEMICAL AND MOLECULAR PROPERTIES

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(Received March 13th, 1979)

Key words: Pyruvate kinase; Subunit structure; Amino acid analysis; Tryptic digestion; (E. coli)

Summary

The two forms of pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) present in *Escherichia coli* have been purified from the same cultures and crystallized. A modified procedure for the purification of type I pyruvate kinase is described.

Molecular weight, subunit structure, amino acid composition, NH_2-terminal amino acid, maps of tryptic peptides and conditions for crystallization have been determined for the two forms.

A comparison of these data shows that the two forms are different proteins, each being a tetramer of identical subunits.

Introduction

The existence of multiple forms of pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) has been reported in animal tissues [1] and in several microorganisms. The molecular mechanisms underlying this multiplicity are widely different: both interconvertible and non-interconvertible forms of the enzyme have been observed.

Some of the convertible forms are conformers of a single protein, which can be 'frozen' either with a bound allosteric effector or free of it; this is the case, for example, of forms A and B of type L pyruvate kinase from liver and possibly of the enzymes from adipose tissue and kidney [2—5]. Alternatively, interconvertible forms are the expression of the aggregation of different numbers of subunits, as in the case of human erythrocytes pyruvate kinase [6].

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In cases of non-interconvertible forms in animal systems, clear evidence of the coexistence of different enzymic proteins within the same tissue is available. In liver, for example, two immunologically and kinetically distinct proteins have been described [7], known as type L and type M pyruvate kinase. But a detailed analysis has shown that the former type is present only in hepatocytes and the latter only in Kupffer cells [8]. It seems reasonable to think that also in other animal tissues where different non-interconvertible forms have been observed, each type of pyruvate kinase is actually present only in a given kind of cell of that tissue.

Among microorganisms, on the other hand, two examples are known where non-interconvertible forms are present within the same cell. In *Escherichia coli* two different pyruvate kinases [9—12] under independent genetic control [13] do coexist in a wide range of nutritional states [9,14,15]. In *Mucor rouxii* three isozymes have been identified (types I, II, III), which are simultaneously present within the filamentous form of the organism in different ratios according to the growth conditions. Type I and III appear to be coded for by different genes, while type II is an hybrid between the former two [16,17].

The two forms observed in *E. coli* differ in kinetics as well as in molecular properties: type I is activated by fructose 1,6-bisphosphate, type II is activated by AMP and by several intermediates of the hexose phosphate pathway. Both forms have been purified [10—12], but the data concerning their molecular properties have been obtained mainly on preparations from different bacterial strains, leaving some doubts on the contribution of strain differences to the overall differences observed.

It is the aim of the present paper to compare some chemical, structural and molecular properties of the two forms of pyruvate kinase extracted from the same bacterial culture.

Materials and Methods

Phospho*enol*pyruvate, tricyclohexylammonium salt, and ADP, sodium salt, were purchased from Sigma (St. Louis, U.S.A.).

Lactate dehydrogenase, NADH and standard proteins for molecular weight estimation were produced by Boehringer (Mannheim, F.R.G.).

Acrylamide, methylenebisacrylamide, *N,N,N',N'*-tetramethylenediamine, 2-mercaptoethanol and *p*-toluenesulphonic acid were obtained from Eastman Kodak Co. (Rochester, U.S.A.).

N-1-Naphthylethylenediamine dihydrochloride and ammonium sulphamate were purchased from BDH Chemicals Ltd. (Poole, U.K.).

Tos-Phe-CH_2Cl-treated trypsin was supplied by Worthington Biochemical Co. (NJ, U.S.A.).

Iodoacetic acid and 5-dimethylamino-1-naphthalenesulphonylchloride (dansyl chloride) were obtained from Fluka A.G. (Buchs, Switzerland).

Sodium dodecyl sulphate was produced by Serva Feinbiochemia (Heidelberg, F.R.G.).

Dextran Blue 2000 and DEAE-Sephadex A 50 were purchased from Pharmacia (Uppsala, Sweden).

DEAE-cellulose (DE-32) and phosphocellulose (P 11) were supplied by

Whatman Biochemicals Ltd. (Springfield Mill, U.K.).

All other chemicals of reagent grade were produced by Merck (Darmstadt, F.R.G.) or by C. Erba (Milano, Italy).

Methods

Protein estimation, polyacrylamide gel electrophoresis with and without sodium dodecyl sulphate, molecular weight estimation by gel filtration, amino-terminal analysis, crystallization and pyruvate kinase assay were performed as previously described [12].

Amino acid analysis. This 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 M 70 Beckman resin, according to Spackman et al. [18], modified by Dévényi [19]. Samples of protein were hydrolyzed in 6 M HCl, in sealed evacuated tubes for 24, 48 and 72 h at 105° C. Tryptophan, phenylalanine, tyrosine, lysine, histidine and arginine were estimated also after acid hydrolysis with *p*-toluenesulphonic acid, according to Liu and Chang [20]. Tryptophan was independently assayed on alkaline hydrolysates according to Basha and Roberts [21].

Carboxymethylation. This was performed as reported by Ronchi et al. [22].

Tryptic digestion. This was carried out on the reduced carboxymethylated enzymes at pH 8.0, for 4 h at 37° C. The trypsin-to-protein ratio was 1/50. At the end of the digestion, the incubation mixture was freeze-dried.

Strain and culture conditions. The strain used was *E. coli* K 12 J53 [23]. Bacterial cultures and crude extracts were obtained as previously described [12].

Type I pyruvate kinase. This was purified according to a modification of the procedure of Malcovati et al. [10]. Crude extracts were applied to a 6 × 35 cm DEAE-cellulose column equilibrated with 5 mM phosphate buffer, 1 mM EDTA, 2 mM mercaptoethanol, pH 7.5 (buffer A). After washing with 2 ml of buffer A, a linear gradient of KCl from 0 to 0.5 M in the same buffer, for a total volume of 1800 ml, was applied to the column. The flow rate was 70 ml/h. Two peaks of pyruvate kinase activity appeared in the effluent [9]: fractions corresponding to the first peak and containing more than 10 units/ml were pooled.

The pool was heated at 55° C for 1 h; the precipitate was removed by centrifugation at 46 000 × *g* for 10 min; the supernatant was dialysed against 10 mM Tris, 1 mM EDTA, 2 mM mercaptoethanol, pH 7.5 (buffer B) and then concentrated to a small volume by ultrafiltration under N_2 pressure in an Amicon ultrafiltration cell equipped with a PM 30 membrane.

The concentrated solution was applied to a 2.5 × 50 cm phosphocellulose column [10], equilibrated in buffer B. After washing with 1 l of the same buffer, the enzyme was eluted with a solution of 2 mM fructose 1,6-bisphosphate in buffer B. The flow rate was 30 ml/h. The enzyme appears in the effluent as a single sharp peak [10]. Fractions containing more than 1 U/ml were pooled, concentrated to less than 1 ml and applied to a 1.0 × 100 cm Bio-Gel A 1.5 M column equilibrated in buffer B. Elution was performed with the same buffer and 2.7-ml fractions were collected. Fractions containing more

TABLE I

PURIFICATION OF THE FRU-1,6-P_2-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	248	47.2	18.7 *	4638 *	0.4 *	1	100
DEAE-cellulose	109	7.2	20.2	2202	2.8	7	47
Heat treatment	104	3.2	20.7	2153	6.5	16	46
Phosphocellulose	71	— **	11.3	802	— **	— **	17.3
Bio-Gel A 1.5 M	8	0.85	75.8	606	89.2	223	13.1

* 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.

** A_{280} was not measured because of the high dilution of protein and interference by fructose 1,6-bisphosphate.

than 40 U/ml were pooled. The purification is summarized in Table I. In most preparations, the enzyme gave a single band in polyacrylamide gel electrophoresis. In some cases, on the contrary, a final additional purification step on DEAE-Sephadex was required. Aliquots of approximately 5 mg of protein, dissolved in 10 mM Tris, 1 mM EDTA, 2 mM mercaptoethanol, 100 mM KCl, pH 8.5 (buffer C), were applied to a 1.6 × 22 cm column of DEAE-Sepharose A 50, equilibrated in the same buffer. Elution was performed with 80 ml of buffer C, followed by a linear gradient of KCl from 0.1 to 0.5 M in the same buffer (total volume of the gradient: 200 ml). Fractions showing constant specific activity (U/A_{280}) were pooled and used for subsequent work.

The specific activity of the above preparations, when assayed as described [12], was 90 U/mg protein. These preparations displayed the same kinetic properties as the enzyme purified by different procedures [10,11].

Type II pyruvate kinase. This was purified as previously described [12]. Since the purification procedures for the two forms share the first step (chromatography of the crude extracts on DEAE-cellulose), they were carried out together on the same material through this step and completed separately.

Results

Electrophoretic mobility

The two forms of pyruvate kinase have been submitted to electrophoresis on polyacrylamide gel at different concentrations. The relative mobilities are given in Table II: at all concentrations tested, the Fru-1,6-P_2-activated form of the enzyme (type I) showed a higher mobility than the AMP-activated form (type II). Coelectrophoresis of the two forms preincubated together for a short time (30 min in 10 mM Tris, 1 mM EDTA, 2 mM mercaptoethanol, pH 7.5, at room temperature) results in two distinct protein bands, each showing the same electrophoretic mobility as either form shows when analyzed independently (Fig. 1).

Both forms of enzyme give a single band when submitted to polyacrylamide gel electrophoresis in sodium dodecyl sulphate (Fig. 2); a subunit molecular weight of 56 000 for type I and of 51 000 for type II was obtained from a plot

TABLE II

ELECTROPHORETIC MOBILITIES OF TYPE I AND TYPE II PYRUVATE KINASE AT DIFFERENT POLYACRYLAMIDE GEL CONCENTRATIONS

Electrophoretic mobility is relative to bromophenol blue.

Gel concentration (%)	Electrophoretic mobility	
	Pyruvate kinase I	Pyruvate kinase II
7	0.440	0.311
8	0.399	0.280
9	0.321	0.217
10	0.277	0.182

of logs of molecular weights of standard proteins versus electrophoretic mobilities.

Molecular weight estimation

Molecular weight of the two forms has been estimated by gel filtration: a value of 225 000 ± 12 000 for type I and of 190 000 ± 10 000 for type II has been obtained.

For both forms of the enzyme the results of molecular weight estimation by

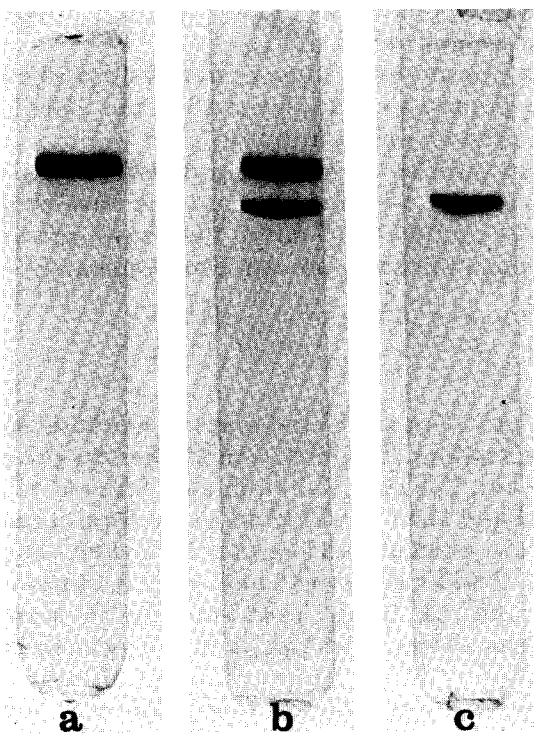

Fig. 1. Coelectrophoresis on polyacrylamide gel of the two forms of pyruvate kinase from *E. coli*. (a) Pyruvate kinase II; (b) pyruvate kinase I and pyruvate kinase II, and (c) pyruvate kinase I.

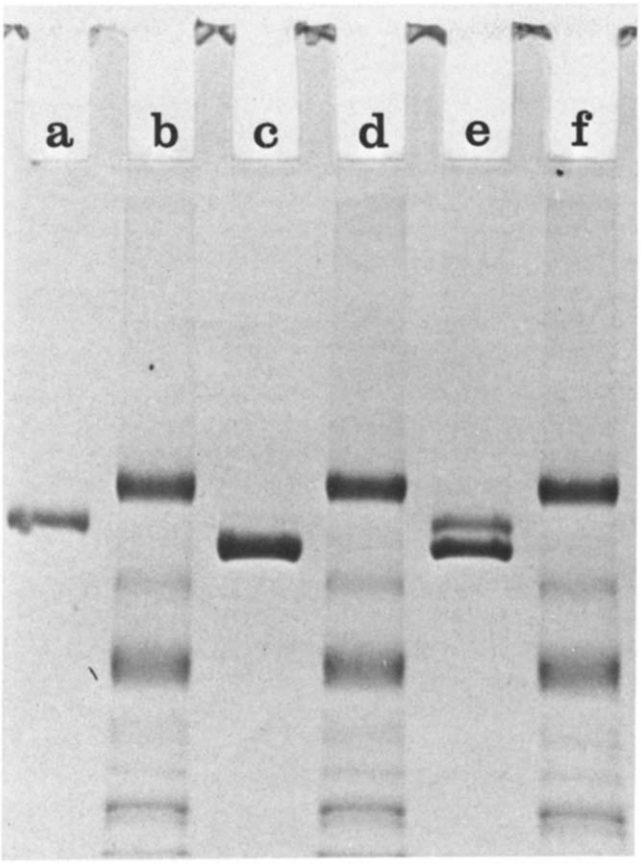

Fig. 2. Polyacrylamide gel electrophoresis in sodium dodecyl sulphate of pyruvate kinases from *E. coli*. (a) Pyruvate kinase I; (b, d, f) standard proteins (bovine serum albumin, aldolase, chymotrypsinogen); (c) pyruvate kinase II; and (e) pyruvate kinase I and II.

polyacrylamide gel electrophoresis are, within experimental error, a quarter of the values obtained by gel filtration, suggesting a tetrameric structure for these proteins, in agreement with the structure of all pyruvate kinases described so far [24].

NH_2-terminal amino acid

A free terminal amino acid can be detected in both forms of pyruvate kinase: it corresponds to serine for type II [12] and to methionine for type I (Fig. 3). The latter result differs from the data of Waygood and Sanwal [11] who found a blocked terminal amino group in their preparation of type I pyruvate kinase from *E. coli* K 12, strain 3000.

Amino acid analysis

The results of the amino acid analysis on both forms of enzyme are shown in Table III. The data are expressed as the nearest whole number of residues of each amino acid/monomer (56 000 daltons in the case of type I and 51 000

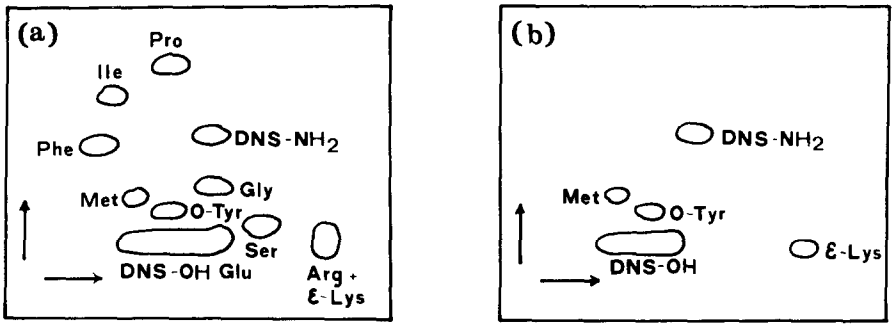

Fig. 3. Bidimensional chromatography of dansyl-amino acids. (a) Cochromatography of an hydrolysate of dansylated pyruvate kinase I and *N*-dansyl derivatives of arginine, serine, glutamic acid, glycine, phenylalanine, isoleucine and proline. (b) Chromatography of the hydrolysate of dansylated pyruvate kinase I alone.

TABLE III

AMINO ACID COMPOSITION OF TYPE I AND TYPE II PYRUVATE KINASE FROM *E. COLI*

Amino acid	Type I				Type II				Integer No.	
	Residue/subunit of molecular weight 56 000			Average (extrapolated)	Residue/subunit of molecular weight 51 000			Average (extrapolated)	Type I	Type II
	24 h [a]	48 h [b]	72 h [b]		24 h [c]	48 h [b]	72 h [b]			
Lys [d]	37.63	38.35	38.87	38.02	28.72	28.70	28.80	28.74	38	29
His [d]	9.18	9.37	9.43	9.27	6.87	7.20	6.82	6.96	9	7
Arg [d]	20.83	21.60	21.20	21.06	29.57	29.64	29.75	29.63	21	30
Asp	57.15	57.35	57.58	57.29	56.89	55.18	57.11	56.46	57	56
Thr [e]	37.27	37.10	36.15	38.15	28.72	27.74	26.35	29.85	38	30
Ser [e]	26.73	26.38	23.77	28.30	26.02	23.33	21.46	28.00	28	28
Glu	51.16	51.32	51.58	51.29	37.62	37.30	37.74	37.56	51	38
Pro	14.86	14.55	14.09	14.62	13.77	14.74	14.18	14.18	15	14
Gly	44.81	43.98	44.27	44.51	43.80	44.73	42.53	43.70	45	44
Ala	45.85	45.25	45.18	45.57	54.21	54.60	54.10	54.29	46	54
Val [f]	43.93	44.85	45.45	45.45	45.41	44.54	47.36	47.36	45	47
Met	15.97	18.64	18.22	17.06	15.19	13.47	15.75	14.86	17	15
Ile [f]	33.13	33.85	34.18	34.18	25.66	25.16	25.14	25.66	34	26
Leu	42.81	43.03	43.54	43.02	43.69	43.32	42.86	43.35	43	43
Tyr [d,f]	6.55	5.85	5.53	6.55	8.23	6.51	6.22	8.23	7	8
Phe [d]	13.52	14.19	13.48	13.65	9.43	8.89	8.50	9.06	14	9
Cys [g]	13.52	—	—	13.52	6.22	—	—	6.22	14	6
Trp [h]				0				0	0	0
Total									522	484

[a] Unless otherwise stated, average of five analyses.
[b] Average of two analyses.
[c] Unless otherwise stated, average of three analyses.
[d] Includes values from hydrolysis according to Liu and Chang [20], for 24 h.
[e] Extrapolated to zero time.
[f] Maximum values taken.
[g] Determined as cysteic acid.
[h] See text.

daltons for type II). The values of serine and threonine are corrected for losses during acid hydrolysis, while valine and isoleucine values are derived from 72 h hydrolysis.

The total number of residues is 522 for type I and 484 for type II.

No tryptophan has been detected in *p*-toluenesulphonic acid hydrolysates of either 1.0 mg of type I pyruvate kinase or 1.1 mg of type II. A negative result has also been obtained when tryptophan was assayed according to Basha and Roberts [21] on 2.0 mg of type I (8.9 nmol) or of type II (10.5 nmol) pyruvate kinase. The absence of tryptophan from type I pyruvate kinase is in agreement with the observations of Waygood and Sanwal [11].

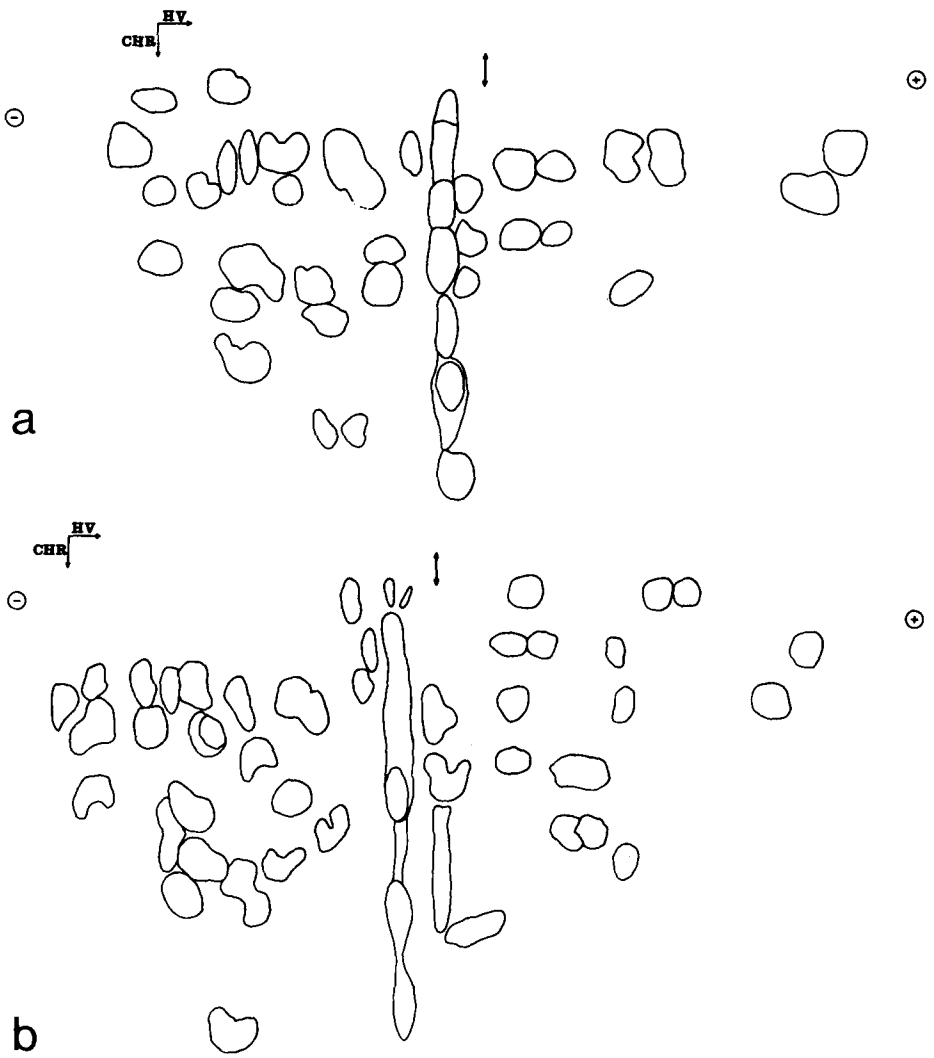

Fig. 4. Peptide maps of tryptic digests of carboxymethylated pyruvate kinases from *E. coli*. Electrophoresis (HV): pH 6.5, 60 V · cm^{-1} 40 min. Chromatography (CHR): acetic acid/*n*-butanol/H_2O/pyridine (6 : 30 : 24 : 20). (a) Pyruvate kinase I; (b) pyruvate kinase II.

Tryptic digestion

Samples of 4.3 mg of pyruvate kinase I and 3.0 mg of pyruvate kinase II were carboxymethylated and subjected to tryptic digestion. The resulting peptides were submitted to fingerprinting. Typical peptide maps are shown in Fig. 4. 40 and 45 spots can be detected in case of type I and type II pyruvate kinase, respectively. On the assumption that both enzymes are tetramers made up of identical subunits, the upper limit for the number of peptides, on the basis of the total number of lysyl and arginyl residues, is 59 and 60, respectively. Since no attempts have been made to resolve neutral peptides, the values obtained in these experiments represent a lower limit for the number of peptides.

In both cases the results are consistent with the existence of a single type of subunit in each protein.

Several differences appear in the two fingerprints in the region of acidic, as well as of basic peptides. Since both high voltage electrophoresis and chromatography of the tryptic hydrolysates of the two forms were run in parallel, the differences observed cannot be ascribed to the processing of the fingerprints. On the other hand, since results of electrofocusing experiments (Galliano, M., unpublished results) suggest the possibility that some deamidation may have taken place in type II pyruvate kinase, only the presence of specific positively charged peptides in this form of the enzyme should be taken into account for discussion of differences between the two forms.

Crystallization

Both forms of pyruvate kinase have been crystallized. While type II crystal-

TABLE IV

EFFECT OF BUFFER COMPOSITION ON THE CRYSTALLIZATION OF THE TWO FORMS OF PYRUVATE KINASE FROM *E. COLI*

All the experiments were performed in buffers containing 0.1 M Tris, 1 mM EDTA, 1 mM mercaptoethanol (buffer D), at different pH values. MPD, 2-methyl-2,4-pentanediol.

	Temp. (°C)	Salting out agent	pH	Result
Type I pyruvate kinase				
Buffer D, no additions	4	60% $(NH_4)_2SO_4$	6.5	Amorphous precipitate
	4	57% $(NH_4)_2SO_4$	7.0—8.0	Crystalline precipitate (prisms)
	4	59% $(NH_4)_2SO_4$	8.5	Mixture of crystals with bad morphology and amorphous precipitate
	25	60% $(NH_4)_2SO_4$	7.3	Crystalline precipitate (prisms)
	25	60% $(NH_4)_2SO_4$	5.8	Crystalline precipitate (needles)
	4	29% MPD	7.0—8.0	Amorphous precipitate
Type II pyruvate kinase				
Buffer D, 0.15 M KCl	4	49% $(NH_4)_2SO_4$	6.8—8.7	Amorphous precipitate
Buffer D, 0.15 M KCl	25	50% $(NH_4)_2SO_4$	6.8—8.7	Amorphous precipitate
Buffer D, 0.15 M KCl	4	none	7.5	No precipitate
Buffer D, 0.15 M KCl	4	none	7.5	Amorphous precipitate
Buffer D, 0.15 M KCl	4	29% MPD	6.6—7.6	Crystalline precipitate

lizes only between pH 6.5 and 7.5, in the presence of K^+ and of agents decreasing the dielectric constant of water [12], type I can give two types of crystals according to the conditions of crystallization (Table IV), but does not crystallize in the conditions in which type II does.

No attempts have been made to determine crystallographic parameters, because of the limited dimensions of the crystals.

Discussion

The gross molecular structures of the two forms of pyruvate kinase extracted from the same culture of *E. coli* appear to be similar. Both forms, in fact, are tetrameric proteins, made up by very similar, if not identical, subunits. This conclusion is supported by the fact that in both cases a single band is detected on polyacrylamide gel electrophoresis in sodium dodecyl sulphate, by the finding of a single aminoterminal residue in each form and by the number of tryptic peptides identified in fingerprints.

Unlike *M. rouxii* [17] and some animal tissues [1,4,25] from which hybrid molecular forms between different types of subunit of pyruvate kinase have been extracted, in *E. coli* no evidence of spontaneous hybrid formation between the two types of subunit has been observed, either in crude extracts [10] or when the purified enzymes are mixed in non-denaturing conditions (Fig. 1). These data suggest a rather stable quaternary structure of the two forms of pyruvate kinase in this organism.

In spite of those gross similarities, several data confirm the conclusion that the two forms of pyruvate kinase are indeed two different proteins, as suggested by kinetic [9—12] and genetic [13] data.

The results of the amino acid analysis of the two forms, showing a higher content in acidic amino acids in type I, are in full agreement with the behaviour of the two forms on electrophoresis (Table II and Fig. 1) and on chromatography on DEAE-cellulose [9].

Since type II has a lower molecular weight than type I and an NH_2-terminal amino acid different from methionine, it might be suggested that the former could arise from type I by proteolytic cleavage of at least a fragment from the NH_2-terminus of the chain. This hypothesis is ruled out by two sets of data: first, the comparison of amino acid composition of the two types shows that the lower molecular weight form has a higher content in arginine, serine, alanine and tyrosine; secondly, the fingerprints of tryptic peptides of the two forms show definite differences. Although the latter observation might not be significant in the case of acidic peptides, owing to the above-mentioned possibility of deamidation having taken place in type II, the fact that positively charged peptides are present only in the fingerprints of this form of enzyme strongly points against the hypothesis of the proteolytic cleavage.

A comparison of the amino acid analysis of pyruvate kinase I (Table III) with the results of Waygood and Sanwal [11] shows that the two sets of data are in agreement within experimental error, once the differences in subunit molecular weight used in the calculations are taken into account. The main difference between the two preparations, in what chemical properties are

concerned, lies in the fact that the NH_2-terminal amino acid was undetectable in Sanwal's preparation, while it has been identified as methionine in ours.

Acknowledgements

This work was supported by a grant from the Italian Science Research Council (C.N.R.). The authors are indebted to Dr. Martino Bolognesi for carrying out the crystallization experiments and to Dr. Lorenzo Minchiotti for the identification of the NH_2-terminal amino acid and for helpful discussion.

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