Characterization of Charge Isomers of Yeast Phosphoglycerate Kinase. Evidence for Intracellular Differences

LARS ARVIDSSON, BIRGITTA SCHIERBECK and MÄRTHA LARSSON-RAŹNIKIEWICZ*

Department of Biochemistry, University of Göteborg and Chalmers Institute of Technology, Fack, S-402 20 Göteborg 5, Sweden

Three electrophoretic components of phosphoglycerate kinase have been isolated from baker's yeast. The isoionic point of the major component is 7.18 at 10 °C. Corresponding values for the minor ones are 6.91 and 7.48, respectively.

There is a difference of one charge-unit between the isomers 1 and 2, and between the isomers 2 and 3. The release of component 3 from the yeast cells appears in contrast to the isomers 1 and 2 to be promoted by an organic solvent, thus suggesting this component to be bound to the cell-membrane.

The amino-terminal amino acid residue appears to be *N*-acetylated serine in each of the three cases. The carboxyl-terminal ends seem to be identical also with -(Ala, Leu, Val, Lys)-Ala-Lys as the ultimate sequence.

From circular dichroism spectra the contents of α -helix and β -structure were estimated to 15 and 40–50 %, respectively. Factors have been determined for transformation and comparison of the specific activities as determined under the various conditions used at different laboratories.

In the last few years intensified studies on phosphoglycerate kinase (ATP: 3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) from different sources have given evidence for enzyme isomers. Larsson-RaŹnikiewicz¹ has found that the yeast enzyme prepared according to the old method of Bücher² contains three enzymatically active electrophoretic components. Also the presence of different forms of the enzyme in the crude yeast autolysate is demonstrated by counter-current distribution technique.^{3,4} Beutler⁵ and Chen *et al.*⁶ have

found three isomers of phosphoglycerate kinase in homogenates from human erythrocytes. In contrast, the purified erythrocyte enzyme seems to consist of only one component.^{7–9} Liver phosphoglycerate kinase^{10,11} purified from human and bovine species give three active components each. Genetic studies including starch gel electrophoresis indicate that *Drosophila melanogaster* contains three condominant alleles¹² for phosphoglycerate kinase and that *Macropus* species have two forms of this enzyme, of which the major component is autosomally controlled.^{13,14}

The preparation method used in the present work is in principal similar but in details different from methods published recently.^{15,16} Three electrophoretic components with phosphoglycerate kinase activity were obtained as previously.¹ The present study was undertaken to ascertain the origin of our different components as others^{15,16} have not noticed any such heterogeneities in their preparations of yeast phosphoglycerate kinase, when tested by gel electrophoresis. Precautions were taken to exclude possible artifacts caused by proteolytic activity and/or organic solvents. Different chemical and physical methods were used to characterize and compare the three phosphoglycerate kinase components obtained. Two of them have in part been characterized earlier.¹

MATERIALS AND METHODS

Reagents. The DEAE cellulose used was Whatman DE 23. Amberlite resin MB 3 was obtained from BDH Chemicals. The carboxypeptidases A, B, and C (DFP-treated) were

* Permanent address (M.L.-R.): Department of Chemistry, Agricultural College of Sweden, S-750 07 Uppsala, Sweden.

products of Worthington Biochemical Corporation. Phenylmethylsulfonyl fluoride came from Sigma Chemical Company. The salt-free DCC-treated crystalline preparation of trypsin was obtained from Miles-Servac. *N*-Acetylserine was a gift from Dr. G. Fölsch (Department of Medical Chemistry, University of Göteborg) and *N*-formylserine was prepared by the method of Sheehan and Yang.¹⁷

Activity measurements. Phosphoglycerate kinase activity was assayed with Bücher's spectrophotometric method² (cf. Ref. 1).

Protein determination. Protein concentrations were determined as absorbance at 280 nm. For pure phosphoglycerate kinase E(1 %, 1 cm) = 5.0 at 280 nm was used.^{2,1}

Isolation of isomers. Autolysis of the yeast cells was carried out in two different ways, IA and IB, respectively, as was the ensuing salt fractionation steps (IIA and IIB). The following steps were identical in both cases. Fig. 1 is representative for a preparation including toluene autolysis (procedure A).

IA. Toluene autolysis. Autolysis of the yeast cells was performed according to Westhead and McLain.¹⁸ The autolysate was centrifuged (432 000 *g* min).

IIA. Precipitation with ammonium sulfate. The pH of the water phase from IA was adjusted to 7.5 with 5 M KOH. Solid ammonium sulfate (70 g/100 ml) was slowly stirred into the solution. The pH decreased to 7.1. The precipitate

was collected by centrifugation (1 296 000 *g* min) and then dissolved in a minimum volume of 0.1 M Tris-Cl buffer (pH 9.5) whereupon it was dialyzed overnight (15 h) against 5 mM Tris-Cl buffer (pH 9.5) at 12 °C.

IB. Freeze autolysis. Two kg of fresh yeast was mixed with an equal quantity of dry ice (w/w) and 50 g of Trizma-base. The mixture was finely dispersed in a Waring Blender and, after melting, 115 ml of conc. ammonia was slowly added during stirring which was continued overnight. The following morning centrifugation was performed (1 296 000 *g* min).

IIB. Fractionated ammonium sulfate precipitation. Ammonium sulfate (30 g/100 ml) was added to the supernatant from IB. After removal of the precipitate by centrifugation, more ammonium sulfate (35 g/100 ml) was added and the pH was adjusted to 7.1. The precipitate was then treated as described above (IIA).

III. DEAE Cellulose chromatography. After adjustment of the pH to 9.5 with 1 M Trizma-base, the solution from II was applied to a DEAE cellulose column (5.7 × 60 cm) equilibrated with 50 mM Tris-Cl buffer (pH 9.5) at 12 °C. The column was washed with 2 l of this buffer, containing 8 mM KCl. Phosphoglycerate kinase was then eluted with 60 mM KCl in the same buffer, and pH was adjusted to 8.0 with HCl, whereupon the solution was concentrated to 25 ml in a Diaflo apparatus.

IV. Gel filtration. The concentrate from III was applied to a Sephadex G-100 column (5.7 × 105 cm), equilibrated with 50 mM Tris-Cl buffer (pH 8.0) at 12 °C. This buffer was also used for the elution procedure. Fractions containing phosphoglycerate kinase activity were collected and concentrated to a volume of about 3 ml.

V. Column electrophoresis. The solution from IV was applied to the top of a water-cooled electrophoresis column with cellulose as supporting medium. The electrophoresis was performed as described earlier.¹

Amino acid analysis. The amino acid compositions were determined as described in Ref. 1. The tryptophan content was analysed spectrophotometrically by the method of Edelhoch.¹⁹

Isoelectric focusing. Electrofocusing was performed on an Ampholine column LKB 8101 using a sucrose gradient. The carrier ampholyte had a pH range of 6.5–7.5. The experiments were mostly carried out as described elsewhere.^{20,21}

Polyacrylamide gel electrophoresis. Sodium dodecylsulfate polyacrylamide gel electrophoresis was run for 180 min (current: 5 mA/gel) at pH 9.5 as described by Maurer.²² Protein zones were made visible after staining with amido black. Gels were destained by putting them into 7 % acetic acid, which contained Amberlite resin MB 3 (after a suggestion from Dr. L. Henderson).

Fig. 1. Isoelectric focusing of phosphoglycerate kinase performed instead of column electrophoresis as the final purification step. The experiment was run for 67 h at 10 °C at 400 V. The (+) and the (−) at the top of the figure indicate the position of the anode and the cathode, respectively. About 60 mg of protein was used. Fraction volumes: 1 ml. — — —, A_{280} ; —, specific activity; x, pH.

Fingerprinting. Tryptic digestion of heat-denatured phosphoglycerate kinase was performed for 7 h at 37 °C in a 0.1 M ammonium bicarbonate buffer (pH 8.8). The ratio of phosphoglycerate kinase to trypsin was 50:1 (w/w). After digestion the protein was lyophilized and subjected to high-voltage paper electrophoresis in a Michl apparatus²³ equipped with a water-cooled coil in the cover. The paper used was Whatman 1 or Whatman 3MM (57 × 42 cm). The pH of the pyridine buffer was 6.5 and the electrophoresis was run for 35 min at 52 V/cm. The chromatography was carried out for 15 h in butanol-acetic acid-water, 3:1:1, (v/v). The chromatograms were stained by the cadmium-ninhydrin method.²⁴ Peptides containing tryptophan residues were identified using Ehrlich's reagent.²⁵

Identification of the terminal amino acids of phosphoglycerate kinase. For determination of the C-terminal amino acids, phosphoglycerate kinase was heat-denatured for 10 min in 0.2 M bicarbonate buffer (pH 8.0), containing 10 % dimethyl sulfoxide. Digestion of the protein with carboxypeptidase A was performed at 37 °C. The ratio of phosphoglycerate kinase to carboxypeptidase was 40:1 (w/w). Dansylation according to Gray²⁶ was performed in sodium dodecylsulfate. The procedure of Yoshida²⁷ was followed for identification of the blocked NH₂-terminal residues.

Circular dichroism measurements. A Cary Model 60 recording spectropolarimeter with circular dichroism attachment was used. The results are expressed in the terms of the mean residue ellipticity, θ , defined as: $\theta = M\theta_{\text{obs}}/10cl$, where θ_{obs} is the observed ellipticity in degrees, M is the mean residue weight, l is the optical pathway in cm, and c is the concentration in g/ml. M was calculated to 107 for the enzyme (*cf.* Refs. 1, 15). No corrections for the refractive index of the solvent was made.

RESULTS AND DISCUSSION

Purification of different electrophoretic components of phosphoglycerate kinase. Isolation of the enzyme according to the above methods gives yields of about 60 %. Vigorous stirring in step IA is necessary to get high amounts of the enzyme.

The elution pattern obtained after column electrophoresis of the material from procedure A is almost identical with the one obtained earlier (Fig. 2 in Ref. 1) when quite a different preparation method was employed.² Three peaks with phosphoglycerate kinase activity occur, one major (named 2) and two minor (named 1 and 3). The material from component 1 (previously¹ called A) was earlier shown to

be heterogeneous in the ultracentrifuge and to possess lower specific activity than the components 2 and 3 (previously¹ called B and C). The present results show that a restrictive collection of the fractions with phosphoglycerate kinase activity is needed after elution of the G-100 column to get a component 1 devoid of contaminating proteins, having molecular weights of 35 000 and 70 000.

The electrophoretic components of phosphoglycerate kinase can be completely separated by isoelectric focusing (Fig. 1). The enzyme content of peak 1 varied from one preparation to another; about 5 % of the protein content of component 2 was obtained as a maximum value. The corresponding value of component 3 was almost constant, 25–30 %, if toluene autolysis of the yeast cells was performed. When freeze autolysis was used only traces of form 3 could be detected by isoelectric focusing. Neither did our attempts to prepare isoenzymes according to Scopes¹⁴ give any component 3. The two purification methods giving this component involve treatment of fresh yeast cells with an organic solvent, ethanol² or toluene. Possible interconversion of the different enzyme forms, induced by such a solvent, was investigated by incubation of pure component 2 with toluene at pH 7.0 and 25 °C for 6 h. No interconversion of the enzyme could, however, be detected by isoelectric focusing. To find out whether an organic solvent facilitates solubilization of component 3 from the yeast cells, the precipitate from step IB (freeze autolysis) was treated with toluene during continuous stirring for 3 h. The supernatant gained threefold in phosphoglycerate kinase activity by this treatment. The enzyme was isolated as described above (the steps IIA–IV), and was finally applied for isoelectric focusing. The preparation contained about the same amount of the two electrophoretic components 2 and 3, the latter corresponding to about 20 % of form 2 totally prepared from the same yeast cells (*cf.* above). These results show that the components 2 and 3 within the cell have different solubility properties, and may suggest that the component 3 occurs bound to the cell membrane, supporting results by Green *et al.*²⁸ who demonstrated that glycolytic enzymes are associated to the membranes of yeast and red blood corpuscles.

Isoelectric focusing. The isoionic points²⁹ of the charge isomers were found to be 6.91, 7.18, and 7.48 for components 1, 2, and 3, respectively, at 10 °C. Treatment with 6 M urea did not change the isoionic points of any of the phosphoglycerate kinase components. Unfolding of the enzyme in urea should normalize possible pK values of amino acid residues being anomalous in the native state.³¹ Hence the present results may indicate that the differences in isoionic points are not due to conformationally induced modifications of the ionization constants. However, we cannot exclude the possibility that the unfolding of the protein molecules in urea is incomplete (*cf.* the unavailability of the carboxyl-terminal amino acid in urea, below).

Amino acid composition and molecular weight. No significant difference in amino acid composition was observed between the three forms of the enzyme. These results and sodium dodecylsulfate polyacrylamide gel electrophoresis suggest the molecular weight of isomer 1 to be in the range of $44\,600 \pm 1600$, earlier determined for the isomers 2 and 3 (Ref. 1).

Specific activities and kinetic parameters. The isolation procedure presented in this paper gives three charged isomers of phosphoglycerate kinase with the same specific activity, 990 U/mg at 25 °C and saturation of both the substrates (*cf.* Ref. 1). To facilitate comparisons of the specific activities determined under various conditions at different laboratories, parallel experiments were performed under the conditions used by Krietsch and Bücher,¹⁵ by Scopes,¹⁶ and by ourselves.¹ A factor of 0.78 was found for conversion of reaction rates at 30 to 25 °C. Estimations showed that the reaction velocities measured at the conditions of Krietsch and Bücher and of Scopes should be multiplied by 1.33 and 0.98, respectively, to be comparable with the activities presented in this paper.

The isomers 1 and 3 appear identical to the isomer 2 concerning the K_m values for $MgATP^{2-}$ and 3-*P*-glycerate, and the typical non-linear Lineweaver-Burk plots at 10 mM Mg^{2+} (*cf.* Ref. 30).

Tests for protease activity during the purification procedure. Kunitz' casein method³¹ indicated a protease activity in step IA corresponding to a trypsin content not exceeding 1 mg/l of the autolysate. This proteolytic activity

Fig. 2. Tryptic finger print map of phosphoglycerate kinase 2. Details of the experiment are given in the section MATERIALS AND METHODS. Solid patches represent Ehrlich positive peptides, cross-hatched patches represent peptides stained yellow, and unfilled patches represent peptides stained red by the cadmium-ninhydrin method. The (+) and the (-) in the figure indicate the anode and the cathode, respectively.

could be completely inhibited by addition of phenylmethylsulfonyl fluoride (*cf.* Ref. 32), a final concentration of 0.5 mM, to the autolysate after toluene removal. The electrophoretic pattern was not affected by the inhibitor treatment.

Fingerprinting. We could not see any differences in the tryptic finger print maps (Fig. 2) of the isomers 2 and 3. The amino acid compositions predict an ideal digest to give about 55 peptides. Around 60 ninhydrin positive spots could be identified for both enzyme components, which is an indication for good tryptic digestions. The same deviations from ideality appear to exist in both cases. Three Ehrlich positive spots were obtained though each protein contains only two tryptophanyl residues.

Carboxyl-terminal and amino-terminal ends. Both in its native state and in 6 M urea the carboxyl-terminal amino acid residue of phosphoglycerate kinase was shown to be unavailable for digestion with the carboxypeptidases A, B, and C. Successful results were obtained

Fig. 3. Liberation of amino acids from phosphoglycerate kinase 2 by carboxypeptidase A. Details of the experimental conditions are given in the section MATERIALS AND METHODS. O, lysine; □, alanine; ●, valine; ▲, leucine.

after heat-denaturation of the enzyme in 10 % dimethyl sulfoxide. The release of amino acid residues during carboxypeptidase A digestion of phosphoglycerate kinase 2 is seen in Fig. 3, which demonstrates that -(Ala, Leu, Val, Lys)-Ala-Lys is the sequence of the carboxyl-terminal end. Corresponding experiments on the enzyme forms 1 and 3 gave identical results.

Attempts to identify the amino-terminal residues by dansylation in dodecylsulfate,³⁶ indicated that these residues are blocked in all the three isomers. Yoshida's method³⁷ for investigation of blocked amino-terminal residues showed that an *N*-acetylated serine

occurs at the terminus in all the three cases (the serine recovery in the acid hydrolysis step was 50 % of the total yield).

Circular dichroism spectra. The circular dichroism spectrum of phosphoglycerate kinase 2 is seen in Fig. 4. The spectra of the isomers 1 and 3 appeared to be identical. Calculations according to Greenfield and Fasman³⁸ give contents of α -helix and β -structure of 15 and 40–50 %, respectively. Bryant *et al.*³⁴ found in crystallographic studies some 86 residues to be involved in helical segments, which would correspond to 20 % of the protein molecule if a molecular weight of 45 000 is presumed.

Fig. 4. Circular dichroism spectrum of phosphoglycerate kinase 2. The experiments were performed at room temperature in 1.0 mM Tris-Cl buffer (pH 7.8). The optical pathway was 1 mm in the 200–250 nm region, and 10 mm in the 250–300 nm region. The corresponding enzyme concentrations were 0.7 mg/ml and 2.5 mg/ml, respectively.

Concluding remarks

If toluene autolysis of the yeast cells is utilized, three electrophoretic components with phosphoglycerate kinase activity can be separated from yeast by column electrophoresis or isoelectric focusing. A similar electrophoretic pattern was earlier obtained when quite a different preparation method was used (*cf.* Ref. 1). In preparations using freeze autolysis or autolysis achieved by ammonia, one of the minor components, named 3, otherwise occurring in about 25 % of the major component, is missing.

The present results demonstrate that after freeze autolysis has been performed and the soluble phase has been removed, component 3 can be released from the ruptured cell fragments by toluene treatment. The yield of this enzyme form was the same as if toluene treatment had been performed on the intact cells, indicating that component 3 is more firmly bound to the cell than the components 1 and 2. An extract obtained by treatment of the yeast cells with, *e.g.* ethanol was in the old preparation method the source of the enzyme. Thus it appears that removal of component 3 from the yeast cells is facilitated by an organic solvent.

Calculations according to Edsall and Wyman²⁵ show that the divergences in isoionic points between the three phosphoglycerate kinase components approximately correspond to one charge-unit per molecule between the forms 1 and 2, and between the forms 2 and 3. For a protein like phosphoglycerate kinase with many of the charged amino acids occurring in such amounts that the error limit for one of these amino acids is ± 1 , it is not possible unambiguously to exclude a difference in charge corresponding to one amino acid. However, the agreeing finger print maps of the enzyme forms are indications, though no proof, of identical sequences. Phosphoglycerate kinase 1 occurs in variable amount, suggesting this component to be a product of component 2, possibly as a result of a split of one amide bond.

Analyses of the terminal amino acids show that proteolytic digestion as a cause of the charge isomers of yeast phosphoglycerate kinase can be excluded.

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