Use of Isoelectric Focusing and a Chromophoric Organomercurial to Monitor Urea-Induced Conformational Changes of Yeast Phosphoglycerate Kinase

By ROBERT A. STINSON

*Department of Pathology, Division of Medical Laboratory Science, University of Alberta,
Edmonton, Alberta T6G 2G3, Canada*

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The effects of urea in concentrations from 0 to 6M on the following properties of yeast phosphoglycerate kinase were studied: the kinetics of inactivation of the enzyme, the spectrum of 2-chloromercuri-4-nitrophenol bound to the single thiol group of the enzyme, the rate of reaction between the mercurial and enzyme, and the isoelectric point. The enzyme was activated by as much as 30% in 1M-urea, and the other data were interpreted as a possible 'tightening' of enzyme structure. The catalytic behaviour of the enzyme in 2M-urea was time-dependent, the initial effects being similar to those in 1M-urea. Polyacrylamide-gel isoelectric focusing of the enzyme in the presence of 2M-urea showed a single species of enzyme with an isoelectric point intermediate between those in 1M- and 3M-urea; a species with an identical isoelectric point was obtained after an 11-day exposure at 4°C to the denaturant at 2M. The enzyme was rapidly inactivated in 3M-urea, with the thiol group fully exposed and the isoelectric point 0.9pH unit higher than in the absence of urea. No further conformational changes could be demonstrated with urea concentrations of 4M or greater. It is suggested that the equilibrium species that exists in 2M-urea has one of two buried lysine residues exposed. The second lysine residue is exposed in 3M or greater concentrations of the denaturant.

I have reported previously (Stinson, 1974) on the use of the chromophoric organomercurial 2-chloromercuri-4-nitrophenol to probe the environment of the single thiol residue of yeast phosphoglycerate kinase (EC 2.7.2.3) and to monitor changes in this environment when substrates bind. I have now used this chromophore to study conformational changes that take place after exposure of the enzyme to urea. The thiol group in the native enzyme is relatively inaccessible, as model thiols and denatured phosphoglycerate kinase react 1000 times faster with the mercurial. Thus a reporter group on this thiol group and the availability of the thiol to reaction with the reporter group should be good monitors for changes in the internal environment of the molecule.

Ui (1971) has suggested that valuable information can be obtained from the change in isoelectric point that occurs when a protein is exposed to denaturing agents such as urea. An amino acid can have a unique environment that results in an unusual pK_a value necessary for its essential role in the catalytic mechanism. In addition, a concentration of acidic or basic residues in the interior of a molecule may be revealed by isoelectric focusing in a strong denaturant. I report here the application of this approach to

yeast phosphoglycerate kinase. The data suggest that the native enzyme contains two buried lysine residues that are uncharged at a pH equal to the isoelectric point of the native enzyme.

Experimental

Materials

Yeast phosphoglycerate kinase was prepared as previously described (Stinson, 1974). The 2-chloromercuri-4-nitrophenol was a generous gift from Dr. D. R. Trentham (University of Pennsylvania, U.S.A.). Carrier ampholytes (pH 7–9) were purchased from LKB-Produkter A.B., Bromma, Sweden. Acrylamide, bisacrylamide and ammonium persulphate, all of high purity, were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. Pig pancreas trypsin (type IX) was from Sigma Chemical Co., St. Louis, MO, U.S.A., as were all other biochemicals. Reagent-grade chemicals were from Fisher Scientific, Montreal, P.Q., Canada. Heparinized human whole blood haemolysed in 9 vol. of 0.01M-KCN was used as a source of haemoglobin. Solutions of technical-grade urea were passed through a mixed-bed ion-exchange resin (Rexyn I-300; Fisher Scientific)

immediately before use. Solutions of 2-chloromercuri-4-nitrophenol were made up in 0.1 M-NaOH. Phosphoglycerate kinase was stored at 4°C in the crystalline state in 4 M-(NH₄)₂SO₄ and dialysed against the appropriate buffer before use. Unless otherwise indicated, the buffer was 50 mM-triethanolamine hydrochloride adjusted to pH 7.5 with NaOH.

Methods

Absorption spectra. These were obtained with either a Cary 16 spectrophotometer at room temperature (22°C) or a Beckman Acta CIII instrument at 30°C.

Phosphoglycerate kinase determination. The enzyme was assayed as previously described (Stinson, 1974) in a Beckman Acta CIII spectrophotometer at 30°C. For protein determinations a mol.wt. of 46000 and an A_{280} of 0.49 for a 0.1% (w/v) solution were used.

Mercurial was standardized by A_{410} readings in 0.1 M-NaOH by using a millimolar absorption coefficient ($\epsilon_{\text{mm}}^{1\text{cm}}$) of 17.4.

To evaluate the stability of phosphoglycerate kinase in urea, enzyme (11.5 µg/ml) was incubated at 22°C in buffer that contained various urea concentrations. At timed intervals 5 µl of sample was removed and assayed in 1.0 ml of assay medium that contained, in addition to substrates and buffer, 50 µg of trypsin. The trypsin was added to prevent the renaturation of the enzyme on dilution into the assay medium. It was not possible to carry out the assays in urea because of the susceptibility of the linking enzyme, glyceraldehyde 3-phosphate dehydrogenase, to the denaturant.

Spectra and pK_a values in urea of phosphoglycerate kinase complexed with 2-chloromercuri-4-nitrophenol. Enzyme (14.4 µM) was treated with 12.8 µM-mercurial in 138 mM-triethanolamine hydrochloride, pH 7.5, and the appropriate urea concentration. When there was no further change in the A_{413} (maximum time was 10 min), the solution was scanned against a blank that contained all components except mercurial. Relative concentrations of the ionized and protonated species were determined at 413 nm ($\epsilon_{\text{mm}}^{1\text{cm}} = 17.4$) and 325 nm ($\epsilon_{\text{mm}}^{1\text{cm}} = 9.3$) respectively.

Reaction rates in urea between 2-chloromercuri-4-nitrophenol and phosphoglycerate kinase. A Durrum stopped-flow instrument was used to monitor, within 5 ms of mixing, the reaction at 22°C between 10 µM-enzyme and 200 µM-mercurial. The reaction was followed at 410 nm in 140 mM-triethanolamine hydrochloride (pH 7.5) in the absence and presence of urea concentrations up to 5 M. The enzyme was exposed to the urea for 5 min before the reaction with mercurial. The reaction rates were displayed on a storage oscilloscope and permanently recorded on Polaroid film. The pseudo-first-order reaction rates determined from the photographs were converted into second-order constants.

Isoelectric focusing. Polyacrylamide gels (4%, w/v) were made and focused at 4°C as previously described (Godolphin & Stinson, 1974; Stinson, 1975), except that NNN'-tetramethylethylenediamine was omitted, and freshly deionized urea, to the desired concentration, was added to the gels. Gels that contained urea were made and focused on the same day. Approx. 8.5 µg of phosphoglycerate kinase, either alone or with an amount of haemoglobin that was readily visible, was added to the cathodal end in the presence of 25% (w/v) sucrose. When focusing was to be done in the presence of urea, the sample was incubated for 1 h at room temperature with the concentration of urea used in the gel and thus applied. All samples were layered below a protective solution of 20% (w/v) sucrose that contained 2% (v/v) Ampholine. Focusing was for 18 h.

Determination of isoelectric points. The basic approach has been previously described (Stinson, 1975). On termination of focusing the gels were removed from the running tubes and sliced into 3.1 mm segments. A minimum of five segments on either side of the estimated position of the enzyme were taken. After pH determination of the segments, phosphoglycerate kinase was located by enzyme assay. A volume of 100 µl of 300 mM-triethanolamine hydrochloride, pH 7.5, containing 6 mM-MgSO₄ and 6 mM-ATP (to facilitate renaturation) was added to each eluate, followed by a further 24 h elution at 4°C. The phosphoglycerate kinase eluted from each segment by the buffer was then assayed as described above. Five segments were also cut on either side of the visible haemoglobin band and their pH was determined. The 'apparent' isoelectric points of phosphoglycerate kinase and haemoglobin determined thus were corrected for the error caused by the pH measurements in urea. Since urea does not alter the isoelectric point of haemoglobin (Ui, 1971), it was used as a pH marker in the absence and in the presence of all urea concentrations. Nine determinations were made in the absence of urea and yielded a value of 7.00 ± 0.03 (s.d.) for the isoelectric point of haemoglobin. At each urea concentration, in each gel, an apparent isoelectric point was determined for haemoglobin as described above. To obtain a corrected isoelectric point the difference between the apparent isoelectric point of haemoglobin and the 'true' value of 7.00 was subtracted from the apparent isoelectric point of phosphoglycerate kinase determined from the pH measurements.

Results

Mercurial spectra and enzyme activities as probes of the urea-induced conformational changes of phosphoglycerate kinase

Complexes of 2-chloromercuri-4-nitrophenol with enzyme were made and exposed to urea concentrations

Fig. 1. Spectral scans of 2-chloromercuri-4-nitrophenol complexed with phosphoglycerate kinase in various concentrations of urea

The spectra were obtained within 10min of mixing the components. Concentrations of urea are indicated beside the lines.

Table 1. Time course for the denaturation of yeast phosphoglycerate kinase with urea

The temperature of incubation was 22°C. For further details see the text.

[Urea] (M)	Time (h)	...	Inhibition (%)			
			0	0.75	1.5	6.5
0			0	0	0	0
1			-30	-8	-7	-6
2			-12	25	25	63
3			61	98	99	99
5			99	99	99	99

up to 5M. Wavelength difference spectra of these solutions were recorded against blanks that lacked only mercurial (Fig. 1). Urea at concentrations up to 5M had little effect on the spectrum of free mercurial at pH7.5. The maximum absorbance, at 402nm for the mercurial alone, was shifted to 410nm in the presence of enzyme, and gross changes in the spectrum are induced by urea, some of which correlate with the activity of the enzyme (Table 1). The enzyme could not be assayed in urea because of the susceptibility of the linking enzyme, glyceraldehyde 3-phosphate dehydrogenase, to the denaturant, and hence, to prevent any renaturation of the enzyme during the assay, 50 μ g of trypsin was added to the cuvette. In the absence of the proteolytic enzyme the kinetic assays were very non-linear. Addition of trypsin was rationalized on the basis that native phosphoglycerate kinase activity is not affected by a 60min exposure to the proteolytic enzyme (R. A.

Stinson, unpublished work), whereas urea-denatured enzyme is much more susceptible to proteolytic digestion. In the presence of trypsin the initial absorbance change with time was linear at all urea concentrations.

The spectrum of the enzyme-mercurial complex is significantly altered by 1M-urea (Fig. 1) and a 30% activation of enzyme also occurs (Table 1). Initially on exposure to 2M-urea there is a 12% activation of the enzyme, but, with time, inhibition occurs. The enzyme lost 61% of its activity immediately on dilution into 3M-urea, and coincident with this was a significant change in the environment of the bound mercurial, as revealed by the spectrum. There is little difference in the spectrum of the complex in 5M-urea compared with that in 3M-urea, but there is an immediate inactivation of the enzyme in 5M-urea. Except in 1M-urea, most of the spectral changes are consistent with a decreasing pK_a of the phenolic hydroxyl group of the bound mercurial as the urea concentration is increased. From the spectra shown in Fig. 1 a pK_a value of 8.20 was calculated in the absence of urea, and 7.36 in the presence of 5M-urea.

Phosphoglycerate kinase reaction with 2-chloromercuri-4-nitrophenol in urea

The second-order rate constants for this reaction in urea concentrations up to 5M and in 3.5mM-sodium dodecyl sulphate are listed in Table 2. To develop a sufficient signal change the reactions in 0, 1M- and 2M-urea had to be carried out in the absence of EDTA (pK_a increase from 6.60) and those

Table 2. *Effect of urea on the rate of reaction between 2-chloromercuri-4-nitrophenol and yeast phosphoglycerate kinase*

Rates were determined at 22°C after the enzyme was exposed to the urea for up to 5 min.

[Urea] (M)	Sodium dodecyl sulphate (3.5 mM)	EDTA (5 mM)	$10^{-3} \times$ Rate constant ($s^{-1} \cdot M^{-1}$)
0	—	—	2.6
1	—	—	1.4
2	—	—	0.9
2	—	+	0.12
3	—	+	800
5	—	+	700
0	+	—	700

in 3M-, 4M- and 5M-urea in the presence of EDTA (pK_a decrease from 8.9). The shielding of the mercurial from the enzyme by EDTA would explain the different rate constants obtained in 2M-urea in the presence and in the absence of the ligand. In any case, the rate of reaction in 2M-urea was similar to that in the absence of urea, but in the presence of 3M-urea the reaction was more than 1000 times faster. The reaction rates in 3M- and 5M-urea and in 3.5 mM-sodium dodecyl sulphate are essentially the same and suggest that the enzyme thiol group is completely exposed under these conditions.

Isoelectric focusing in the presence of urea

Cyanates were eliminated when stock aqueous solutions of urea were passed through a mixed-bed ion-exchange resin (Rexyn I-300) at 4°C immediately before casting the gels. Subsequent cyanate formation was minimized by limiting the prefocusing incubation time of protein and urea to 1 h at room temperature and by focusing at 0°C. The pH measurements of the eluate from the gel segments were done at room temperature. However, the temperature error introduced in the isoelectric point so determined is unlikely to be significant, since Ui (1971) has reported a low temperature coefficient of dissociation for the carrier ampholytes.

A correction to the pH determinations in urea was made with the use of haemoglobin (see under 'Methods'). The correction determined for gels containing 6M-urea resulted in a lowering of the apparent isoelectric point of phosphoglycerate kinase by 0.18 pH unit.

Fig. 2 is a photograph of gels in which phosphoglycerate kinase alone has been focused in the presence of various urea concentrations. The enzyme shows two bands in both 0 and 1M-urea, but in 2M-urea and higher concentrations only one band is evident. Arvidsson *et al.* (1976) have reported the presence of up to three electrophoretically separable species of yeast phosphoglycerate kinase. The minor

Fig. 2. *Polyacrylamide-gel isoelectric focusing of phosphoglycerate kinase in various concentrations of urea* Gels (a)–(g) contain 0, 1M-, 2M-, 3M-, 4M-, 5M- and 6M-urea respectively.

band in the gels shown in Fig. 2 developed on storage of the crystalline enzyme in 4M-(NH₄)₂SO₄ at 4°C and could be the result of a deamination reaction. Enzyme that had been denatured by dialysis against 6M-urea in buffer and then renatured by dialysis against buffer containing 2.4 mM-MgATP²⁻ also showed two bands when focused in the absence of urea, with isoelectric points identical with those of the native enzyme. In addition, when the renatured enzyme was focused in the presence of 3M-urea, only one protein-stainable band was present, with an isoelectric point similar to that obtained with enzyme that had been focused in 3M-urea but had not been denatured and renatured. When native enzyme was adsorbed on a Sepharose column with ATP covalently bound, and subsequently eluted with 6 mM-3-phosphoglycerate, the protein still exhibited two bands on isoelectric focusing in the absence of urea. This suggests that both protein bands are active; the specific activity of the preparation is in agreement with this conclusion. The presence of urea in the gels altered the gradient only slightly and hence the positions of the bands in the different gels roughly correspond to their isoelectric points. Fig. 2 demonstrates a dramatic increase in the isoelectric point of phosphoglycerate kinase as the urea concentration

Fig. 3. Plot of corrected isoelectric point of phosphoglycerate kinase against the urea concentration

The values are the averages of three to seven determinations, with the range of values indicated by vertical bars.

Fig. 4. Amount of phosphoglycerate kinase activity, as a percentage of the amount applied, recovered after focusing in various urea concentrations followed by elution and renaturation with Mg^{2+} -ATP

For details, see under 'Methods'. Each value is the average of three to seven determinations, with the ranges of values indicated by vertical bars.

is increased from 1 M to 3 M, and Fig. 3 is a plot of the isoelectric points determined at each urea concentration. In all concentrations of urea except 2 M, the enzyme focused into a discrete band. A sample of phosphoglycerate kinase exposed to 2 M-urea for 11 days at 4°C and then subjected to isoelectric focusing also focused as a smear and had an isoelectric point identical with that of the sample incubated for only 1 h in 2 M-urea. Although the protein focused in 2 M-urea was very diffuse (Fig. 2), the isoelectric points determined on different gels by enzyme assays were quite precise (Fig. 3).

A compilation of the percentage recoveries of enzyme activity after focusing, elution and renaturation revealed a possible activation or protection of the enzyme in 1 M-urea (Fig. 4). It is noteworthy that the enzyme is activated by up to 30% in 1 M-urea (Table 1), and the amount of activity recovered from the gels focused in 1 M-urea is 70% higher than in the absence of the denaturant. The enzyme is stable in 1 M-urea, as no detectable activity loss could be shown after 1 week at 4°C. The enzyme did not focus sharply in 2 M-urea (Fig. 2) and three times as much length of gel was necessary to obtain 95% of the recovered enzyme activity in 2 M- than in 3 M-urea.

Discussion

All of the approaches used in the present study to monitor various aspects of the enzyme's structure indicate changes in 1 M-urea. The enzyme is activated (Table 1), the spectrum of bound mercurial is altered (Fig. 1), the enzyme thiol group appears less available to reaction with mercurial (Table 2), and the recovery of catalytic activity from gels after isoelectric focusing is increased (Fig. 4). These observations may indicate a 'tightening' of the molecule that results in greater stability and activity. The lower pK_a of the phenolic hydroxyl group in the presence of urea may also contribute to the lower rate of reaction between enzyme and mercurial in 1 M-urea. In 2 M-urea the enzyme is initially more catalytically efficient, with a sheltered thiol group, but with time an inactive enzyme with a higher isoelectric point forms (Fig. 3). The enzyme structure(s) present after several hours of exposure to 2 M-urea appear to remain unchanged, as a focusing pattern identical with that in Fig. 2 is obtained after up to 11 days in 2 M-urea. The enzyme in 3 M-urea is altered very rapidly, as evidenced by a 1000-fold increase in thiol reactivity and an increase in isoelectric point of 0.6 pH unit over the equivalent values in 2 M-urea.

The isoelectric point of phosphoglycerate kinase in 6 M-urea was 8.24, an increase from 6.94 in the absence of urea (Fig. 3). Arvidsson *et al.* (1976) reported no effect of 6 M-urea on the isoelectric point of phosphoglycerate kinase, but the unavailability of the C-terminal amino acid in urea under their

conditions suggested that the enzyme was not denatured. The difference of 1.3 units reported herein is very unusual and does not result from the loss of a tightly bound anion, since extensive dialysis against 6M-urea did not alter the isoelectric point of the renatured enzyme focused in the absence of urea. Also, the isoelectric point of the renatured enzyme focused in 3M-urea was identical with that of native enzyme, also focused in 3M-urea. Such an anion could be the substrate for the enzyme, 1,3-diphosphoglycerate, which Johnson *et al.* (1975, 1976) have shown to be tightly bound to the enzyme.

Of five proteins that Ui (1971) focused in the absence and presence of urea, only human serum albumin had an isoelectric point in urea that was different by 0.3 pH unit or more from its isoelectric point in the absence of urea. Ui (1971) determined the isoelectric point of ribonuclease in 6M-urea to be 0.25 unit higher than in the absence of urea, and interpreted this increase as the 'normalization' of a lysine residue in the presence of the denaturant. Of the nine proteins that Solaman & Williamson (1971) investigated, only with bovine albumin was the difference between the isoelectric points in the absence and in the presence of 6M-urea greater than 0.3 unit. The increase in isoelectric point of this protein from 5 to 6 in urea was attributed to the exposure of approximately seven basic groups normally buried in the native molecule.

For the purpose of estimating the number of lysine residues, or their equivalent, that must be buried in native yeast phosphoglycerate kinase to account for the increased isoelectric point, theoretical isoelectric-point calculations were made. If one assumes that phosphoglycerate kinase has (1) a total of 80 aspartic plus glutamic residues (Larsson-Raźnikiewicz, 1970), 22 of which are estimated to be amides, (2) 44 lysine residues (Scopes, 1973), (3) other residue frequencies as listed by Scopes (1973) and pK_a values given by Tanford (1968), then a theoretical isoelectric point of about 7.0 can be calculated (the experimental value reported herein is 6.94). If the calculations are repeated using 46 lysine residues, then a value very close to 8.2 is obtained. It is feasible, then, that the denaturation of yeast phosphoglycerate kinase by 6M-urea exposes two lysine residues that at a pH equal to the isoelectric point of the protein are not protonated as would be predicted from accepted pK_a values. The amount of energy required to bury an amino group with an intrinsic pK_a of 10.5 in the

protein such that at its native isoelectric point of 6.94 it remains uncharged is a minimum of 20.1 kJ (4.8 kcal). Thus for two lysine residues 40.2 kJ (9.6 kcal) is required.

The wide range of pH values over which phosphoglycerate kinase is active (Krietsch & Bücher, 1970) suggests that the active site of the enzyme may be well shielded from the external medium and thereby may have associated with it residues with unusual pK_a values. Chemical inhibition studies led Markland *et al.* (1975) to postulate that three lysine residues are 'essential' for catalytic activity of yeast phosphoglycerate kinase. Partial substrate protection of activity suggested the presence of lysine residue(s) at the active site. If phosphoglycerate kinase does take on a stable conformation in 2M-urea that is different from its conformations in 1M- and 3M-urea, then it is possible that only one of the buried lysine residues is exposed in 2M-urea. This would account for the isoelectric point being intermediate between those in 1M- and 3M-urea.

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