The refolding of denatured rabbit muscle pyruvate kinase

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The refolding of rabbit muscle pyruvate kinase after denaturation by guanidine hydrochloride was studied. On dilution of the denaturing agent, enzyme activity is only partially regained. The extent of regain of activity is dependent on protein concentration, showing a marked decrease at higher concentrations. The failure to regain complete activity appears to be related to the formation of inactive aggregates, which can be separated from active enzyme by gel filtration. Insoluble aggregates can be partially re-activated after solubilization in guanidine hydrochloride. Changes in the circular-dichroism and fluorescence spectra during refolding suggest that a partially folded, inactive species is formed rapidly; this differs from native enzyme in being more susceptible to proteolysis by trypsin.

Pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) from rabbit muscle consists of four subunits, each of M_r about 59000 (Kayne, 1973). A high-resolution X-ray-crystallographic structure of the enzyme from cat muscle has been published (Stuart *et al.*, 1979), but the lack of amino acid-sequence data has precluded further interpretation of the electron-density map. The unfolding and refolding of the enzyme from rabbit muscle has been studied by various workers (Cottam *et al.*, 1969; Johnson *et al.*, 1969; Doster & Hess, 1981). Cottam *et al.* (1969) showed that denaturation of the enzyme in 4M-urea or 4M-GdnHCl, followed by removal of denaturing agent by dialysis or gel filtration, led to recovery of 35–50% of enzyme activity. In a further study, Johnson *et al.* (1969) investigated the effects of various parameters, such as temperature, pH, protein concentration, presence of various metabolites etc., on the extent of recovery of activity after denaturation by GdnHCl, and defined optimum conditions for this process. More recently, Doster & Hess (1981) suggested that at intermediate concentrations (~2M) of urea the enzyme undergoes a reversible expansion and that the 'active dimer' reported in earlier work (Cottam *et al.*, 1969) was in fact an expanded tetramer with a diffusion coefficient smaller than that of the native enzyme. Cardenas *et al.* (1977) and Porter & Cardenas (1980) have undertaken studies on the refolding of denatured bovine muscle pyruvate kinase.

In the present paper we examine two particular aspects of the refolding of rabbit muscle pyruvate kinase after denaturation by GdnHCl. Firstly we explore why incomplete regain of activity is observed, and secondly we use sensitivity to proteolysis (Price & Stevens, 1982) to examine the nature of the product(s) formed during refolding. The results are consistent with a pathway of refolding in which partially folded inactive species are rapidly formed; these then slowly rearrange to yield some active enzyme and some aggregated inactive material.

Experimental

Materials

Pyruvate kinase was isolated from rabbit skeletal muscle as described by Kayne (1971) or was purchased as an $(\text{NH}_4)_2\text{SO}_4$ suspension from Boehringer. The preparations have a specific activity of 180 μmol of pyruvate formed/min per mg of protein at pH 7.6 and 20°C, as measured by the coupled assay with lactate dehydrogenase (Kayne, 1971). Concentrations of enzyme were determined spectrophotometrically by using a value of $A_{280} = 0.54$ for a 1 mg/ml solution and a value of 237000 for the M_r of the tetramer (Kayne, 1973). Creatine kinase was isolated from rabbit skeletal muscle as described previously (Bickerstaff & Price, 1978).

The monopotassium salt of ADP, the disodium salt of NADH, rabbit muscle lactate dehydrogenase, hog muscle lactate dehydrogenase and bovine erythrocyte carbonic anhydrase were purchased from Boehringer. Bovine pancreatic trypsin [treated with 1-chloro-4-phenyl-3-L-tosyl-

Abbreviations used: GdnHCl, guanidine hydrochloride; SDS, sodium dodecyl sulphate.

amidobutan-2-one ('TPCK') was obtained from Worthington via Millipore (U.K.). Trypsinogen, cytochrome *c*, the monocyclohexylammonium salt of phosphoenolpyruvate, polyethylene glycol 4000 and GdnHCl (Aristar grade) were obtained from BDH Chemicals. Soya-bean trypsin inhibitor, bovine serum albumin, a cross-linked bovine serum albumin preparation, phenylmethanesulphonyl fluoride, tosyl-L-arginine methyl ester and dithiothreitol were purchased from Sigma. Sepharose 4B and Blue Dextran 2000 were purchased from Pharmacia (G.B.). Glutaraldehyde [purissimum, 25% (w/v) aqueous solution] was purchased from Fluka. Dimethyl suberimidate dihydrochloride was purchased from Aldrich.

Methods

The denaturation and refolding of rabbit muscle pyruvate kinase were performed under conditions similar to those described by Porter & Cardenas (1980) in their studies on the bovine muscle enzyme. Enzyme was denatured by incubation for 15 min at 0°C in 50 mM-Tris/HCl buffer, pH 7.5, containing 4 M-GdnHCl and 0.1 M-2-mercaptoethanol. Refolding was initiated by a 40-fold dilution of the denatured enzyme into 'refolding buffer' [50 mM-Tris/HCl (pH 7.5)/0.5 M-sucrose/0.1 M-KCl/5 mM-MgCl₂/5 mM-dithiothreitol] at 20°C. Samples were removed at stated times for assay of enzyme activity (Kayne, 1971). In some experiments, GdnHCl was removed by dialysis against 'refolding buffer' rather than by dilution. In those experiments in which the cross-linking of pyruvate kinase was studied, phosphate buffers were employed instead of Tris buffers because of the expected side reaction of the latter with the cross-linking agent. The denaturing buffer contained 0.1 M-potassium phosphate, pH 7.5, rather than Tris/HCl, and the 'refolding buffer' consisted of 0.1 M-potassium phosphate buffer, pH 7.5, containing 0.5 M-sucrose, 5 mM-MgCl₂ and 5 mM-dithiothreitol.

SDS/polyacrylamide-gel electrophoresis was performed as described by Weber *et al.* (1972). Proteins used as *M_r* markers were bovine serum albumin, creatine kinase, carbonic anhydrase, trypsinogen, soya-bean trypsin inhibitor and cytochrome *c*.

The sensitivity of the enzyme to trypsin during refolding was studied by SDS/polyacrylamide-gel electrophoresis by the method described by Price & Stevens (1982) in studies on the refolding of creatine kinase. Enzyme samples were incubated with trypsin (normally either 10% or 100% by weight of enzyme) for 1 min at 20°C, then SDS was added to a final concentration of 0.1% and the samples were immediately transferred to a boiling water bath. After incubation at 100°C for 2 min, the samples were allowed to cool to room temperature and 0.3 mM-phenylmethanesulphonyl fluoride was added

during cooling. Control experiments of the type described by Price & Stevens (1982) showed that trypsin is rapidly inactivated by this procedure. The effect of trypsin on enzyme activity during refolding was investigated as described by Price & Stevens (1982).

Cross-linking of pyruvate kinase by glutaraldehyde was performed by the procedure of Hermann *et al.* (1981). After cross-linking, the mixture was analysed by SDS/polyacrylamide-gel electrophoresis, with the various cross-linked bovine serum albumin species (monomer, dimer, trimer and tetramer) as *M_r* markers.

Fluorescence spectra were recorded at 20°C with a Perkin-Elmer MPF 3A spectrofluorimeter. Spectra were corrected by subtracting the signal from identical solutions not containing protein.

Circular-dichroism spectra were recorded at 20°C in a JASCO J-500A spectropolarimeter equipped with a JASCO DP-500 N Data Processor.

Results

Regain of enzyme activity during refolding

Representative time courses of the regain of activity after dilution of the denaturing agent are shown in Fig. 1(a). After a short lag period, maximal activity is regained over a period of about 1½ h. There is no significant increase in activity over a further 2 h period. The regain of activity is not enhanced by inclusion of substrate (phosphoenolpyruvate or ADP at 1 mM concentration) in the refolding mixture. However, omission of 0.5 M-sucrose or of the reducing agents from the denaturing or refolding mixture leads to a lower regain of activity (approx. 30% activity regained at a protein concentration of 40 µg/ml). The time course of refolding in phosphate buffer is very similar to that in Tris buffer. These findings are broadly in agreement with those reported by Johnson *et al.* (1969).

The lack of complete regain of activity during refolding is not due to the residual concentration of GdnHCl during refolding, since GdnHCl at 0.1 M (the residual concentration during refolding) had no effect on the activity of native enzyme incubated at 20°C in 'refolding buffer' over a period of 1 h. In addition, the regain of activity is not increased by lowering the residual concentration of GdnHCl to 0.05 M. As shown in Fig. 1(a) and in more detail in Fig. 1(b), protein concentration has a pronounced effect on the extent of regain of activity. At protein concentrations above about 20 µg/ml in the refolding mixture, the regain of activity shows a steady decline; the same effect is noted at lower concentrations. Fig. 1(b) also shows that there is considerable variation between duplicate refolding experiments. We can only conclude that such variations

Fig. 1. Regain of activity of pyruvate kinase after denaturation

Enzyme was denatured in 4M-GdnHCl and then diluted 1:40 into refolding buffer as described in the Experimental section. (a) Representative time courses of regain of activity. The enzyme concentrations during refolding are: ○, 20 µg/ml; ●, 40 µg/ml; △, 78 µg/ml. (b) Regain of activity (measured after 2h refolding) as a function of concentration of enzyme during refolding. The means $\pm$ S.D. for at least four determinations at each concentration are shown.

must arise from small differences in the manner of initiating the refolding by dilution of the denaturing agent, since they are considerably greater than the variations between duplicate enzyme assays ($\pm 3\%$). In view of these variations we have not been able to undertake a detailed analysis of the kinetics of re-activation.

At protein concentrations higher than about 70 µg/ml, visible turbidity develops during the refolding of denatured enzyme. In experiments at even higher concentrations of protein (550 and 710 µg/ml) in which GdnHCl is diluted by dialysis against 200 vol. of refolding buffer, extensive precipitation of protein occurs and very little activity ($\leq 4\%$) is observed in the supernatant. It should be noted that removal of GdnHCl by dialysis rather than by dilution is not a factor leading to the low regain of activity, since there is no difference in the regain of activity between the two procedures at a lower enzyme concentration (70 µg/ml).

Characterization of refolded pyruvate kinase

Kinetic parameters of refolded enzyme. The kinetic properties of native enzyme and enzyme refolded at a concentration of 40 µg/ml were

Fig. 2. Gel filtration of refolded pyruvate kinase. An enzyme sample was concentrated as described in the text and applied to a column (33 cm $\times$ 1 cm) of Sepharose 4B. Fractions (1.2 ml) were collected and analysed for (○) A₂₈₀ and (●) enzyme activity. In separate experiments, the void volume of the column was determined to be 12 ml and the elution volume of native pyruvate kinase to be 23 ml.

compared. K_m values for ADP and phosphoenolpyruvate were identical in the two cases (0.73 mM, 0.16 mM respectively). This indicates that, although the extent of reactivation is only 45% of the control activity, the active enzyme formed is identical with native enzyme by this criterion.

Gel filtration of refolded enzyme. Enzyme allowed to refold at a concentration of 70 µg/ml regains about 25% activity after 2h. A 24 ml portion of such a mixture was concentrated by dialysis against 20% (w/v) solution of polyethylene glycol 4000. During the concentration procedure some of the protein precipitated, and this was removed by centrifugation (1000g for 5 min). The remainder (representing 70% of the original protein) was applied to a column (33 cm $\times$ 1 cm) of Sepharose 4B and was eluted with refolding buffer containing no dithiothreitol. The elution profile is shown in Fig. 2. Some material is eluted at the void volume of the column; some is eluted at the position corresponding to the elution volume of native pyruvate kinase and some is eluted in later fractions; only the material in the second peak shows enzyme activity. The sample applied to the column is clearly heterogeneous and consists of high- M_r aggregates and some material of low M_r , as well as material of the M_r of native enzyme. The specific activity of material in the second peak (Fig. 2) is 140 units (μ mol/min)/mg, which is similar to that obtained for a sample of native enzyme subjected to this concentration/gel-filtration procedure.

Re-activation of precipitated enzyme. Pyruvate kinase (460 $\mu\text{g/ml}$) was denatured and then dialysed against 200 vol. of refolding buffer. As described above, extensive precipitation occurred and only a very low proportion (3.5%) of activity was regained. After centrifugation, the precipitated pellet was redissolved in denaturing buffer and, after 15 min incubation at 0°C, diluted 40-fold into refolding buffer. After 2 h of refolding 18% of the activity of a non-denatured control sample had been regained. Although this value is somewhat lower than the usual extent of re-activation (45%) at this protein concentration (40 $\mu\text{g/ml}$), this experiment clearly demonstrates that the precipitated protein has the potential for at least partial re-activation, as has been previously observed for pig muscle lactate dehydrogenase (Rudolph *et al.*, 1979). There was no increase in the extent of re-activation when the redissolved pellet was incubated in denaturing buffer for 3 h before dilution.

Structural changes during refolding of pyruvate kinase

Structural changes were monitored by two techniques: protein fluorescence and c.d.

Fluorescence. Native pyruvate kinase shows a fluorescence emission maximum at 333 nm when the excitation wavelength is 290 nm (Fig. 3). This spectrum is not affected by the presence of 0.1 M-GdnHCl (the residual concentration during refolding). Enzyme that has been incubated in the presence of 4 M-GdnHCl for 15 min at 0°C shows a smaller

Fig. 3. Fluorescence emission spectra of pyruvate kinase

All spectra were recorded at an enzyme concentration of 40 $\mu\text{g/ml}$ at 20°C with excitation at 290 nm. (1), Native enzyme in refolding buffer; (2), enzyme denatured by incubation in buffer containing 4 M-GdnHCl for 15 min at 0°C; (3), enzyme diluted 1:40 into refolding buffer after denaturation in 4 M-GdnHCl (the spectrum was recorded 1 min after dilution).

fluorescence, with the emission maximum shifted to 345 nm (Fig. 3). On dilution of the denaturing agent to a concentration of 0.1 M, there is a very rapid change in the fluorescence spectrum. Within 1 min of dilution, the intensity and emission maximum of fluorescence are very similar to those of the native enzyme (Fig. 3). There is no further change in the spectrum over a period of 1 h. The very rapid fluorescence changes (corresponding to changes in the environments of tryptophan side chains) occur before any significant regain of activity has occurred and point to the rapid accumulation of at least partially folded intermediates during the refolding process (Teipel & Koshland, 1971*a,b*). Analogous results were obtained at a variety of enzyme concentrations (10–70 $\mu\text{g/ml}$) and also when denaturation and refolding were performed in

Fig. 4. Circular dichroism of pyruvate kinase

(a) Spectra of pyruvate kinase in the absence of GdnHCl (○) and in the presence of 0.1 M- and 4 M-GdnHCl. (b) Changes in $[\theta]_{\text{m.r.w.}}$ at 225 nm as a function of concentration of enzyme during refolding. (○) and (●) represent the values of $[\theta]_{\text{m.r.w.}}$ measured after 30 s and 60 min of refolding respectively. The means $\pm$ s.d. of three determinations at each concentration are shown. The values of $[\theta]_{\text{m.r.w.}}$ at 225 nm corresponding to denatured and native enzyme were -700 and -9000 degrees $\cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ respectively.

Fig. 5. Densitometer traces after SDS/polyacrylamide-gel electrophoresis of pyruvate kinase

In each case 16 μg of protein was applied to 7.5%-polyacrylamide gels, and the direction of migration towards the positive pole is from left to right. The gels were stained with Coomassie Blue, and the ordinate represents A_{545} . The horizontal scale bar corresponds to the separation between species of M_r 60 000 and 35 000 as determined from the mobilities of the standard marker proteins listed in the Experimental section. The region corresponding to M_r less than 35 000 includes staining owing to trypsin and is not shown. (a) Native enzyme (40 $\mu\text{g}/\text{ml}$) incubated in the absence (i) or presence (ii) of trypsin (40 $\mu\text{g}/\text{ml}$) for 1 min at 20°C. (b) Enzyme during refolding (40 $\mu\text{g}/\text{ml}$) incubated in the presence of trypsin (4 $\mu\text{g}/\text{ml}$) for 1 min at 20°C. (i), (ii) and (iii) represent samples treated with trypsin after 1, 15 and 60 min refolding respectively.

phosphate rather than in Tris buffer (see the Experimental section).

Circular dichroism. The c.d. spectra of native pyruvate kinase and of enzyme incubated in the presence of 4 M-GdnHCl for 15 min at 0°C are shown in Fig. 4(a). These spectra show that in the native enzyme there is a pronounced minimum at approx. 220 nm, characteristic of the presence of α -helix (Chen *et al.*, 1974); this minimum is absent with the denatured enzyme. At 0.1 M, GdnHCl has no effect on the native enzyme. By using the reference value of $[\theta]_{m.r.w.}$ at 225 nm for helical proteins (Chen *et al.*, 1974), the helical content of pyruvate kinase can be calculated to be approx. 32%, a value comparable with that determined by optical rotatory dispersion (Kayne & Suelter, 1968).

The changes in $[\theta]_{m.r.w.}$ at 225 nm that occur during the refolding of pyruvate kinase are summarized in Fig. 4(b). Within 30 s there is a change in $[\theta]_{m.r.w.}$ from -700 to approx. -5000 at each concentration, corresponding to approximately half the total difference in $[\theta]_{m.r.w.}$ at 225 nm between native and denatured enzyme. Subsequently, a slower change in $[\theta]_{m.r.w.}$ at 225 nm occurs, the amplitude of which depends on the concentration of protein during refolding. In general terms there is a correlation between the limiting change in $[\theta]_{m.r.w.}$ at 225 nm and the extent of re-activation as a function of protein concentration over this range of concentrations (compare Figs. 1b and 4b).

Susceptibility of pyruvate kinase to proteolysis during refolding

In our earlier studies on the refolding of creatine kinase (Price & Stevens, 1982) it was shown that measurements of the susceptibility of the enzyme to proteolysis provided a useful method for examining whether or not folded structures accumulated during the refolding of the denatured enzyme.

Native pyruvate kinase at 40 μg is not affected by incubation with 40 $\mu\text{g}/\text{ml}$ of trypsin/ml for 1 min at 20°C, as judged by the lack of effect on enzyme activity and on the size of the M_r -59 000 peak on SDS/polyacrylamide-gel electrophoresis (Fig. 5a). Pyruvate kinase that has been denatured by incubation in 4 M-GdnHCl for 15 min at 0°C is completely degraded by trypsin under these conditions; there is no evidence for any remaining species of M_r greater than 12 000 on SDS/polyacrylamide-gel electrophoresis. Similar results are

(c) Enzyme during refolding (40 $\mu\text{g}/\text{ml}$) incubated in the presence of trypsin (4 $\mu\text{g}/\text{ml}$) for 1 min at 20°C. (i), (ii) and (iii) represent samples treated with trypsin after 1, 15 and 60 min refolding respectively.

obtained when the trypsin concentration is lowered to $4\mu\text{g/ml}$, namely, native enzyme is not affected, but denatured enzyme is completely degraded.

Samples of pyruvate kinase that had been allowed to refold (at $40\mu\text{g/ml}$) for periods of 1, 15 and 60 min were treated with trypsin ($4\mu\text{g/ml}$) for 1 min at 20°C and then analysed by SDS/polyacrylamide-gel electrophoresis. The results (Fig. 5*b*) show that within 1 min of refolding there is a large quantity of material that is much more resistant than the denatured enzyme to trypsin; this observation is consistent with the hypothesis that a folded structure is formed rapidly during refolding. However, the material formed after 1 min of refolding differs from native enzyme in that a second peak is observed on electrophoresis, corresponding to a M_r of about 54 000. It would appear that the rapidly formed folded structure is not as compact as native enzyme, with one (or possibly more than one) site for proteolytic attack exposed near one end of the polypeptide chain. In the later samples (15 and 60 min) the susceptibility to proteolysis comes to resemble more closely that of the native enzyme, as shown by the decrease in the size of the M_r -54 000 peak. The differences between the rapidly formed structure and native enzyme are shown more clearly if the concentration of trypsin is raised to $40\mu\text{g/ml}$ (Fig. 5*c*).

After 1 min of refolding, the material treated with trypsin shows a small amount of intact polypeptide chain, but there is extensive proteolysis to produce not only the fragment of M_r 54 000 noted in Fig. 5(*b*) but also a rather broad band of material in the M_r range down to about 35 000 or lower. In the later samples, the susceptibility to proteolysis comes to resemble that of the native enzyme, but the species that can be proteolysed to produce the M_r -54 000 fragment persists to some extent (Fig. 5*c*).

The susceptibility of the enzyme during refolding to proteolysis by trypsin was also demonstrated by the following experiment. Enzyme ($40\mu\text{g/ml}$) after various periods of refolding was treated with trypsin ($40\mu\text{g/ml}$) for 1 min at 20°C . Phenylmethanesulphonyl fluoride was added to a final concentration of 1.9 mM and the sample was then left for 60 min before assay. Native enzyme is not affected by trypsin under these conditions, whereas denatured enzyme diluted into a refolding buffer containing trypsin regains less than 0.1% of the activity of a non-denatured control sample. Enzyme refolded for periods of 1, 15 and 60 min before trypsin treatment regains 1%, 20% and 35% activity respectively. These values should be compared with the 45% regain of activity after periods of 60–120 min for control samples not treated with trypsin (Fig. 1). Thus the species formed after a short period of refolding is susceptible to proteolysis, since treatment with trypsin impairs its subsequent ability to form

active enzyme. The susceptibility to proteolysis decreases after longer periods of refolding. These conclusions are in agreement with those reached from SDS/polyacrylamide-gel electrophoresis (Fig. 5). Direct comparison between the two sets of results cannot be made, since (i) different properties are being monitored and (ii) addition of phenylmethanesulphonyl fluoride does not lead to immediate inactivation of trypsin, so that the effective period of proteolysis is longer than 1 min. Control experiments showed that complete inactivation of trypsin towards the substrate tosyl-L-arginine methyl ester requires 10 min incubation with 1.9 mM-phenylmethanesulphonyl fluoride. In spite of these reservations, the differences in susceptibility to proteolysis between native and refolding enzyme are clearly demonstrated, as is the inability of cleaved enzyme to refold to yield active enzyme.

In an additional experiment, the effect of trypsin ($40\mu\text{g/ml}$) on the activity of enzyme ($40\mu\text{g/ml}$) at particular time points during refolding was measured, with controls included as described previously (Price & Stevens, 1982). Samples of enzyme were withdrawn from the refolding mixture and treated with trypsin for 1 min at 20°C . At 1 min after addition of 2.3 mM-phenylmethanesulphonyl fluoride, the samples were assayed. There was no detectable effect of trypsin on the activity during refolding, suggesting that there is no accumulation of any trypsin-sensitive material with enzyme activity during the refolding. It appears that activity only arises from species that resemble native enzyme in terms of their sensitivity to trypsin.

Essentially identical conclusions were drawn from analogous experiments performed on enzyme refolding at a concentration of $10\mu\text{g/ml}$.

Cross-linking studies on pyruvate kinase

Cross-linking studies were performed in an attempt to monitor the changes in quaternary structure during refolding. Such an approach has been used successfully in studies on lactate dehydrogenase (Hermann *et al.*, 1981). Cross-linking of pyruvate kinase was performed in phosphate refolding buffer (i.e. 0.1 M-potassium phosphate, pH 7.5, containing 0.5 M-sucrose, 5 mM-MgCl₂ and 5 mM-dithiothreitol). Studies with the native enzyme showed that it is very difficult to cross-link quantitatively all four subunits of the tetramer. By using reaction with 1% (w/v) glutaraldehyde for 2 min the percentages of protein as tetramer, dimer and monomer were 72, 19 and 9 respectively. The percentage of tetramer was increased to 86 by using 5% (w/v) glutaraldehyde. Since the tetrameric enzymes lactate dehydrogenase and yeast phosphoglycerate mutase are each completely cross-linked by 1% (w/v) glutaraldehyde (Hermann *et al.*, 1981; Price & Jaenicke, 1982), the forcing conditions

required to cross-link pyruvate kinase may reflect either the arrangement of the subunits, or the availability of reactive side chains, or both. In this connection, it may be relevant that X-ray-crystallographic data (Stammers & Muirhead, 1975) show that the four subunits are arranged as a pair of dimers, with the contacts between the pairs of subunits related by one dyad being much less numerous than those between pairs of subunits related by the other two dyads.

Samples of pyruvate kinase (40 µg/ml) during refolding were taken and treated with 5% (w/v) glutaraldehyde for 2 min. Analysis of the products by SDS/polyacrylamide-gel electrophoresis showed that most of the material formed large aggregates, which did not enter the pores of the gel. Of the small amount of protein that did enter the gel, it was clear that after 1 min of refolding only monomeric species existed, whereas in samples taken at later times (after 5, 15 and 60 min of refolding) there was much less monomeric and an increasing amount of dimeric and tetrameric material. However, because of the problems caused by aggregate formation, the data were not suitable for a detailed kinetic analysis of the type undertaken, for example, for lactate dehydrogenase (Hermann *et al.*, 1981). Attempts to improve the results by variation in reaction conditions proved unsuccessful.

Experiments using dimethyl suberimidate as a cross-linking agent (Bickerstaff *et al.*, 1980) also proved unsuccessful, since very little material was observed in the bands corresponding to cross-linked tetramers of native enzyme, despite using high concentrations of reagent and prolonged reaction times.

Discussion

Unlike creatine kinase (Bickerstaff *et al.*, 1980), pyruvate kinase does not refold after denaturation by GdnHCl with complete regain of activity. The inability to regain full activity is associated with the formation of inactive material, which can be separated from active enzyme by gel filtration. The turbidity observed on refolding at high enzyme concentrations and the appearance of material at the void volume of the Sepharose column (Fig. 2) indicate that high- M_r aggregates are formed; these have also been observed during the refolding of other enzymes, including pig muscle lactate dehydrogenase (Zettlmeissl *et al.*, 1979), yeast phosphoglycerate mutase (Sugimoto *et al.*, 1966) and bovine muscle pyruvate kinase (Porter & Cardenas, 1980). The precipitated material formed during refolding at high concentrations of pyruvate kinase is, however, capable of at least partial re-activation after solubilization in 4 M-GdnHCl and subsequent dilution. Partial re-activation of insoluble

aggregates by this procedure has also been observed for pig muscle lactate dehydrogenase (Rudolph *et al.*, 1979).

Studies of protein fluorescence (Fig. 3), c.d. (Fig. 4) and susceptibility to proteolysis (Fig. 5) suggest that major structural changes to yield partially folded inactive species occur very rapidly in the refolding of denatured pyruvate kinase. It appears that a certain fraction of these subsequently forms inactive aggregates. This fraction is presumably higher at protein concentrations higher than about 20 µg/ml (see Fig. 1b), thereby decreasing the percentage regain of activity. A decrease in re-activation at low concentrations of enzyme (Fig. 1) has been previously noted in other cases (e.g. pig heart lactate dehydrogenase; Rudolph *et al.*, 1977) and may arise from the instability of intermediates during refolding under conditions where associative processes of second or higher order are very slow.

Although the material formed after 1 min of refolding has a very similar fluorescence spectrum to that of native enzyme, it differs in terms of secondary structure, probably containing about half the helical content of native enzyme, and in that it contains one or more bonds susceptible to proteolysis by trypsin. These bonds could correspond to regions of the polypeptide chain which link the domains in the structure of native pyruvate kinase (Stuart *et al.*, 1979).

Unfortunately, it has not proved possible to assess the rate of regain of the tetrameric structure of the enzyme in the refolding process. The cross-linking method is not successful because of the formation of large amounts of aggregates under the conditions required for cross-linking of the subunits of native pyruvate kinase. Using the ability to form hybrids as a measure of quaternary structure, Cardenas *et al.* (1977) concluded that the tetrameric structure of bovine muscle pyruvate kinase is regained only slowly during refolding. Our observations from the limited cross-linking data suggest that this may also be the case for rabbit muscle pyruvate kinase. It is possible that the hybridization approach might be successfully applied to this enzyme.

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