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The unfolding and refolding of pig heart fumarase

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The unfolding of pig heart fumarase in solutions of guanidinium chloride (GdnHCl) has been examined. Loss of activity occurs at lower concentrations of GdnHCl than the structural changes detected by fluorescence or c.d. After denaturation, regain of activity can be observed provided that a reducing agent (dithiothreitol) is present and that the concentration of GdnHCl is lowered by dialysis rather than by dilution. The regain of secondary structure occurs with high efficiency even when little or no activity is recovered.

INTRODUCTION

Fumarase (fumarate hydratase, EC 4.2.1.2) from pig heart is a tetrameric enzyme of M_r 194000 (Beeckmans & Kanarek, 1977). The enzyme is thought to occur in the matrix of the mitochondria, although there is some evidence that it, along with other tricarboxylic acid-cycle enzymes, may bind to the mitochondrial inner membrane (Srere, 1982). By using covalently immobilized enzyme, evidence has been found for interactions between fumarase and other enzymes of the cycle, notably malate dehydrogenase and citrate synthase (Beeckmans & Kanarek, 1981).

Like most mitochondrial enzymes, fumarase is synthesized as a precursor in the cytosol and subsequently imported into the organelle. As part of a series of experiments in which we have studied the folding and assembly of several mitochondrial enzymes (West & Price, 1988, 1990; West *et al.*, 1990), we have investigated the unfolding and refolding of fumarase. In earlier work (Teipel & Koshland, 1971; Teipel & Hill, 1971), it had been reported that some regain of fumarase activity after denaturation was observed on dilution of guanidinium chloride (GdnHCl) and that this could be enhanced by inclusion of substrate (L-malate). However, in view of the reported improvement in stability of the enzyme prepared by affinity chromatography (Beeckmans & Kanarek, 1977), it seemed worthwhile to investigate the unfolding and refolding of fumarase in more detail. In this paper, we report that loss of fumarase activity occurs at much lower concentrations of GdnHCl than the structural changes observed by fluorescence or c.d. Regain of activity after denaturation is possible provided that dithiothreitol is present, and that the concentration of GdnHCl is lowered by dialysis rather than by dilution. The structural changes on refolding have also been examined.

EXPERIMENTAL

Pig heart fumarase, prepared by the method of Beeckmans & Kanarek (1977), was purchased as an $(NH_4)_2SO_4$ suspension from Sigma. The preparation was at least 95% homogeneous as judged by Coomassie Blue staining after SDS/PAGE (Laemmli, 1970). The subunit M_r (48000) was in agreement with previous determinations (Beeckmans & Kanarek, 1977).

Before use, the enzyme was dialysed extensively against 50 mM-sodium phosphate buffer, pH 7.3. When necessary, the concentration of the enzyme was increased by dialysis against a 20% (w/v) solution of poly(ethylene glycol) (M_r approx. 8000) in 50 mM-sodium phosphate buffer, pH 7.3. Poly(ethylene glycol) was purchased from Sigma.

The concentrations of solutions of enzyme were determined by the Coomassie Blue binding method (Sedmak & Grossberg, 1977), which was found to be more reliable and sensitive than spectrophotometric determination at 280 nm. The validity of the dye-binding method was confirmed by amino acid analysis by using the published amino acid composition data (Beeckmans & Kanarek, 1977). Kanarek & Hill (1964) had previously mentioned the problems caused by turbidity and light-scattering in the accurate spectrophotometric determination of fumarase concentrations.

Enzyme activity was assayed spectrophotometrically at 250 nm by using a volume of 1 ml of 50 mM-L-malate in 50 mM-sodium phosphate buffer, pH 7.3, at 25 °C. In terms of the units described by Kanarek & Hill (1964), the specific activity of the enzyme was found to be 31500 units/mg, in close agreement with the value (33000 units/mg) quoted by Beeckmans & Kanarek (1977). Our value for the specific activity corresponds to 390 μmol of fumarate formed/min per mg of protein (Bock & Alberty, 1953).

C.d. spectra were recorded at 20 °C on a JASCO J-600 spectropolarimeter. Molar ellipticity values were obtained by using a value of 110 for the mean residue weight (Beeckmans & Kanarek, 1977). The secondary-structure content was determined by applying the CONTIN procedure at 1 nm intervals over the range 190–240 nm (Provencher & Glöckner, 1981).

Fluorescence spectra were recorded at 20 °C in a Perkin–Elmer MPF3A fluorimeter.

GdnHCl (Ultrapure grade) was purchased from Gibco BRL, Paisley, Scotland, U.K. The concentrations of solutions were checked by refractive-index measurements (Nozaki, 1972).

RESULTS

Secondary structure of fumarase

The far-u.v. c.d. spectrum of pig heart fumarase is shown in Fig. 1. As determined by the CONTIN procedure (Provencher & Glöckner, 1981) over the range 190–240 nm, the percentages of α-helix and β-sheet structure are 57 ± 1.6 and 26 ± 2.2 respectively. Previous optical-rotatory-dispersion measurements had been interpreted in terms of an α-helical content of about 50% (Kanarek & Hill, 1964). At present there are no high-resolution X-ray structural data on fumarase with which to compare these estimates.

Unfolding of fumarase by GdnHCl

The unfolding of the enzyme was monitored in a variety of ways.

Abbreviation used: GdnHCl, guanidinium chloride.

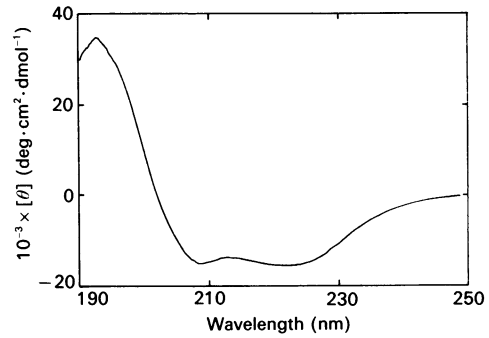

Fig. 1. Far-u.v. c.d. spectrum of fumarase

The spectrum was recorded at 20 °C in 50 mM-sodium phosphate buffer, pH 7.3. The enzyme concentration was 50 μg/ml and the path-length 0.1 cm.

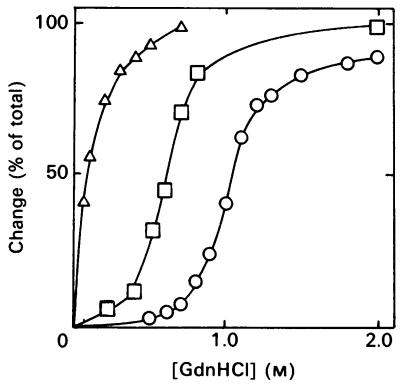

Fig. 2. Unfolding of fumarase by GdnHCl

Unfolding was monitored by changes in (△) enzyme activity, (□) fluorescence at 325 nm and (○) ellipticity at 225 nm as described in the text. Solutions were incubated for 15 min at 20 °C in 50 mM-sodium phosphate buffer, pH 7.3, containing 1 mM-dithiothreitol in the presence of the indicated concentration of GdnHCl. Concentrations of enzyme were (△, ○) 30 μg/ml and (□) 23 μg/ml. In each case the changes are expressed relative to the total change observed between 0 and 4 M-GdnHCl, at which point the changes were essentially complete.

Loss of activity. Fumarase (30 μg/ml) was incubated at 20 °C in the presence of GdnHCl in 50 mM-sodium phosphate buffer, pH 7.3, containing 1 mM-dithiothreitol; samples (20 μl) were removed for assay, with the same concentration of GdnHCl present in the assay mixture as in the original incubation. The loss of activity after 15 min incubation as a function of [GdnHCl] is shown in Fig. 2. From these data it is clear that the integrity of the active site is very easily disturbed by low concentrations of the denaturing agent (i.e. 0.1 M-GdnHCl causes 55% loss of activity). If GdnHCl is not included in the assay mixture, the losses of activity are much less. Thus, for example incubation with 0.5 M-GdnHCl for 15 min causes 41% and 93% losses of activity when GdnHCl is absent from or present in the assay mixture respectively. This difference is presumably due to rapid refolding in the assay mixture of at least a portion of the denaturated fumarase in the former case.

Changes in fluorescence. When excited at 290 nm, fumarase shows an emission maximum at 325 nm characteristic of tryptophan side chains buried in the interior of the protein, away from the aqueous solvent. On addition of GdnHCl there is a decrease in the fluorescence and a shift of the emission maximum towards the red. Thus in the presence of 4 M-GdnHCl, the emission maximum was at 353 nm, a value characteristic of tryptophan side chains fully exposed to the solvent, and the fluorescence at 325 nm was 25% that of the enzyme in the absence of GdnHCl. The changes in fluorescence at 325 nm after 15 min incubation in GdnHCl solutions containing 1 mM-dithiothreitol are shown in Fig. 2.

Changes in far-u.v. c.d. When fumarase was incubated in the presence of increasing concentrations of GdnHCl containing 1 mM-dithiothreitol, there was a decline in the far-u.v. c.d. signal, reflecting the loss of secondary structure. The changes in $[\theta]_{225}$ (which broadly measure α-helical content) after 15 min incubation are shown in Fig. 2.

Changes in quaternary structure. The quaternary structure of fumarase was monitored by gel filtration on Sepharose 4B (under the conditions described in the legend to Fig. 5). In the absence of GdnHCl, the elution volume of fumarase was 18 ml; this was increased to 23 ml in the presence of 0.5 M-GdnHCl. The latter value corresponded to the elution volume of yeast phosphoglycerate kinase (M_r 45000), indicating that at this concentration of GdnHCl fumarase is essentially dissociated into subunits. At higher concentrations of GdnHCl, where loss of secondary and tertiary structure is more evident, the behaviour of fumarase on gel filtration was more difficult to interpret. Thus, for instance, in the presence of 2 M-GdnHCl the elution volume was 19 ml. It is likely that, under such conditions, the behaviour reflects the extensive unfolding of the subunits, leading to a decrease in elution volume compared with that of a compact globular structure (Andrews, 1970; Mann & Fish, 1972).

Comparison of the various measurements of unfolding. From Fig. 2 it is clear that the changes in activity in the enzyme are observed at much lower concentrations of denaturing agent than are the changes in tertiary structure (as judged by fluorescence), which in turn occur at lower concentrations of GdnHCl than the changes in secondary structure (far-u.v. c.d.). These observations are consistent with the pattern noted previously for a number of enzymes, including creatine kinase and glyceraldehyde-3-phosphate dehydrogenase (Tsou, 1986).

Time-dependence of unfolding. The data shown in Fig. 2 refer to measurements made after 15 min incubation in the presence of various concentrations of GdnHCl. At concentrations of GdnHCl of 0.4 M and below, or 1 M and above, there was no detectable time-dependence of the values of the various parameters depicted in Fig. 2 (i.e. the values observed after 1 min and 60 min incubation did not differ significantly).

In this 'intermediate' region of [GdnHCl], however, time-dependent changes were observed in the various measurements of unfolding, although it should be noted that the ranges of concentration of GdnHCl at which such effects were observed were not identical for all three parameters. Changes in activity and fluorescence were observed, for instance, at 0.5 M- and 0.7 M-GdnHCl; however, the changes in c.d. were not seen at the lower of these concentrations. Selected data are shown in Fig. 3. In each case the changes are complete within 30 min, and no additional change is seen over a further 90 min.

There was no significant effect of omitting dithiothreitol from the incubation mixtures on the measurements of unfolding of the enzyme.

Refolding of fumarase after denaturation in GdnHCl

After incubation for 15 min in GdnHCl, the refolding of fumarase was attempted either by dilution into or by dialysis against 50 mM-sodium phosphate buffer, pH 7.3. In the dilution

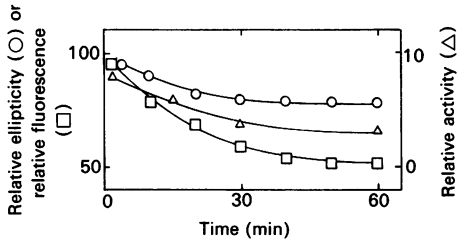

Fig. 3. Time-dependence of unfolding of fumarase by intermediate concentrations of GdnHCl

(△), (□) and (○) refer to changes in enzyme activity, fluorescence and ellipticity as described in Fig. 2. In each case the value corresponding to the enzyme in the absence of GdnHCl is 100. Note the different scale used for the values of enzyme activity.

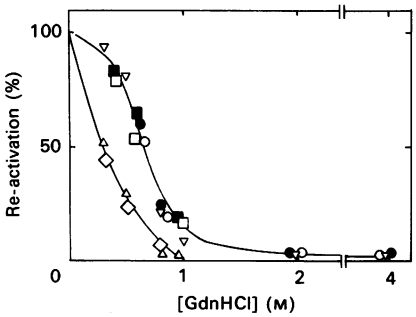

Fig. 4. Re-activation of fumarase after unfolding by GdnHCl

Enzyme was incubated for 15 min at 20 °C in 50 mM-sodium phosphate buffer, pH 7.3, containing 1 mM-dithiothreitol, then diluted into buffer at 20 °C containing dithiothreitol to give a residual concentration of GdnHCl of 0.02 M. Enzyme activity was measured 1 h after dilution. In each case re-activation is expressed relative to a control sample incubated in the absence of GdnHCl. (▽), (○) and (□) refer to enzyme concentrations (after dilution) of 2.5, 5 and 13 μg/ml respectively, and (●) and (■) to 5 and 13 μg/ml respectively with 5 mM-L-malate included in the dilution buffer. (△) and (◇) refer to enzyme concentrations of 2.5 and 13 μg/ml respectively with no dithiothreitol included in either the unfolding or re-activation steps.

procedure the residual GdnHCl was 0.02 M; in the dialysis procedure the residual concentration was shown by refractive-index measurements to be less than 0.01 M.

The results of applying the dilution procedure are shown in Fig. 4. It is clear that (*a*) there is a marked inability of the enzyme to regain activity when the initial concentration is of greater than about 1 M, and (*b*) omission of dithiothreitol leads to a lower regain of activity. The extent of regain of activity could not be increased by increasing the time of incubation or by increasing the concentration of enzyme in the refolding mixture. Essentially similar data to those shown in Fig. 4 were observed when samples were assayed after 24 h incubation in the refolding mixture, or when the final concentration of enzyme in this mixture was varied from 1 to 17 μg/ml. In addition, there was no effect of including L-malate (5 mM) in the refolding mixture (Fig. 4).

When fumarase denatured in GdnHCl was subjected to dialysis to remove the denaturant, a substantial proportion of activity could be regained (Table 1). From Table 1 it is clear that the presence of dithiothreitol is crucial to this regain. A further set of experiments showed that the presence of the reducing agent was necessary in the refolding step (i.e. during dialysis) rather than in the denaturation step, and that L-malate (5 mM) could not substitute for dithiothreitol in bringing about re-activation. Essentially similar data were obtained when the concentration of enzyme was 20 or 10 μg/ml, i.e. within the range of concentrations where the dilution procedure did not lead to regain of activity at an initial [GdnHCl] > 1 M.

From the data in Table 1, the regain of secondary structure (far-u.v. c.d.) is seen to be almost 100% efficient under all circumstances. However, the regain of tertiary structure (as judged by both the emission maximum and intensity) is much less complete in the absence of dithiothreitol than in its presence, and this behaviour is found to parallel the regain of activity. This implies that the integrity of thiol groups is required for correct folding and that this integrity is very easily damaged in the unfolded state.

Table 1. Refolding of fumarase after denaturation in GdnHCl

Denaturation was performed by incubating the enzyme for 15 min at 20 °C with the stated concentrations of GdnHCl and dithiothreitol. Dialysis was performed at 4 °C for 18 h against 500 vol. of 50 mM-sodium phosphate buffer, pH 7.3. The final concentration of enzyme was 30 μg/ml. Values shown are the means of duplicate measurements with less than 10% variation in the values of activity, fluorescence (F_{325}) and molar ellipticity. The precision of the emission maximum is estimated at ±1 nm.

Sample	Activity (% of native)	F_{325} (% of native)	Emission maximum (nm)	$[\theta]_{225}$ (% of native)
Native enzyme	100	100	325	100
Enzyme + 1.5 M-GdnHCl + 1 mM-DTT*				
Before dialysis	0	28	345	22
After dialysis	62	94	325	98
Enzyme + 1.5 M-GdnHCl				
Before dialysis	0	28	345	22
After dialysis	3	72	333	96
Enzyme + 4 M-GdnHCl + 1 mM-DTT*				
Before dialysis	0	25	353	8
After dialysis	48	79	325	95
Enzyme + 4 M-GdnHCl				
Before dialysis	0	25	353	8
After dialysis	1	67	335	96

* 1 mM-dithiothreitol (DTT) was included in both the unfolding incubation mixture and the buffer against which the sample was dialysed.

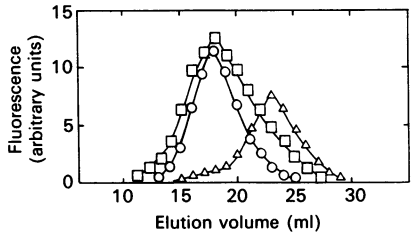

Fig. 5. Gel filtration of native and refolded fumarase

Samples (1 ml) of enzyme (approx. 30 μg/ml) were applied to a column (1.8 cm^2 × 10 cm) of Sepharose 4B eluted with 50 mM-sodium phosphate buffer, pH 7.3; 1 ml fractions were collected. Protein was monitored by fluorescence with excitation and emission wavelengths of 290 and 325 nm respectively. (○) refers to native enzyme; (△) and (□) refer to enzyme denatured by 4 M-GdnHCl and refolded by dialysis in the absence and presence of 1 mM-dithiothreitol respectively. In a separate experiment, the elution volume of yeast phosphoglycerate kinase was determined to be 23 ml.

The ability of the enzyme to regain its quaternary structure after refolding was examined by gel filtration on Sepharose 4B. As shown in Fig. 5, enzyme which had been denatured and refolded in the presence of dithiothreitol had regained the native tetrameric structure, as judged by its elution volume (18 ml). By contrast, enzyme which had been denatured and refolded in the absence of dithiothreitol and which had regained little, if any, activity (Table 1) was eluted at 23 ml, corresponding to the position of elution of yeast phosphoglycerate kinase (M_r 45000). This indicates that under these conditions the enzyme remains in a monomeric state after dialysis.

DISCUSSION

In this paper we have demonstrated that the changes in catalytic activity, secondary structure and tertiary structure of fumarase occur over different concentration ranges of GdnHCl (Fig. 2). This is similar to the type of behaviour reported (Tsou, 1986) for other enzymes and is taken to reflect the sensitive nature of the structure of the active site. The results of gel filtration indicate that in the presence of 0.5 M-GdnHCl, the enzyme is dissociated into subunits, although no great loss of native secondary or tertiary structure has occurred. These results indicate that the catalytic activity of fumarase depends on the tetrameric structure of the enzyme, a conclusion consistent with that reached by Teipel & Hill (1971) using sedimentation-velocity measurements under somewhat different conditions. On refolding, there is efficient regain of secondary structure and the regain of tertiary structure is more marked than that of catalytic activity (Table 1); this is in accord with current models of protein folding (Jaenicke, 1987; Creighton, 1990), in which formation of secondary structure precedes that of 'compact intermediate' and finally catalytically active states.

We have been able to observe some of the features previously reported for the unfolding and refolding of fumarase (Teipel & Hill, 1971; Teipel & Koshland, 1971). Thus we have observed a time-dependence of inactivation at intermediate concentrations of GdnHCl (Fig. 3). Teipel & Hill (1971) noted that activity was slowly lost at concentrations of GdnHCl less than 0.5 M. However, they observed much less inactivation than in the present paper, presumably because they did not include GdnHCl in the assay mixture, and this allowed at least partial refolding to occur before the rate of product formation was recorded. Like Teipel & Hill (1971), we have observed that dialysis of the denatured enzyme against a buffer containing a reducing agent can lead to substantial regain of activity (Table 1). However, unlike Teipel & Koshland (1971), we have been unable to observe re-activation by dilution of the denatured enzyme with initial GdnHCl concentrations above 2 M or to observe any enhancing effect of L-malate on this re-activation. It is possible that the differences between our data and the earlier results may arise from small differences between the enzyme preparations, which, as noted by Beeckmans & Kanarek (1977), are reflected in slight differences in specific activity and composition and pronounced differences in stability on incubation at pH 7.3.

Slow structural transitions at intermediate concentrations of denaturant (Fig. 3) have been observed in other systems (see, e.g., Zettlmeissl *et al.*, 1981; Tsou, 1986). The nature of these transitions remains to be explored, although clearly relatively small structual rearrangements are involved. In some cases *cis* $\rightleftharpoons$ *trans* isomerization of proline peptide bonds may also occur (Zettlmeissl *et al.*, 1981; Brandts & Lim, 1986). Presumably in the presence of these intermediate concentrations of denaturing agents a number of conformational states are kinetically accessible to the enzyme.

In common with other mitochondrial enzymes which we have studied, e.g. glutamate dehydrogenase (West & Price, 1988), aspartate aminotransferase (West & Price, 1990) and citrate synthase (West *et al.*, 1990), fumarase did not regain activity on dilution of the denaturated enzyme, provided that the initial concentration of GdnHCl was higher than about 1.5–2 M. For fumarase, the ability to regain activity ran roughly in parallel with the changes in tertiary structure detected by fluorescence (compare Figs. 2 and 4). However, using the dialysis procedure to lower the GdnHCl concentration led to substantial re-activation. The difference between the two methods is not due to the different temperatures used, since a control experiment showed that dilution into buffer at 4 °C led to no increase in re-activation compared with 20 °C. There has been little systematic study of the different procedures used to bring about refolding of denatured proteins, but it is probable that the success of the dialysis procedure in the present case arises from the gradual lowering of the GdnHCl concentration so that 'correct' structural adjustments can occur. By contrast, when the concentration of GdnHCl is lowered suddenly (e.g. by dilution), the enzyme may be prevented from acquiring its proper tertiary structure by kinetic-energy barriers. A number of examples of irreversible unfolding of proteins at critical concentrations of denaturants have been reported (see, e.g., London *et al.*, 1974; Ghélis & Yon, 1982; Mitraki *et al.*, 1987).

The relevance of the findings reported in this paper to the folding and assembly of fumarase *in vivo* remains to be established. The dependence of the re-activation on the refolding conditions implies that special mechanisms may exist to ensure correct folding *in vivo*. In the light of current data on the import and assembly of mitochondrial and chloroplast proteins, it is possible that 'chaperone' proteins may play a role in this folding process (Cheng *et al.*, 1989; Ellis & Hemmingsen, 1989; Ostermann *et al.*, 1989).

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