Comparison of the activity and conformation changes of lactate dehydrogenase H_4 during denaturation by guanidinium chloride

Yong-Ze MA and Chen-Lu TSOU*
National Laboratory of Biomacromolecules, Institute of Biophysics, Academia Sinica, Beijing 100080, China

The inactivation and unfolding of lactate dehydrogenase (LDH) during denaturation by guanidinium chloride (GuHCl) under diverse conditions have been compared. Unfolding of the native conformation, as monitored by fluorescence and c.d. measurements, occurs in two stages with increasing GuHCl concentrations, and the inactivation approximately coincides with, but slightly precedes, the first stage of unfolding. The enzyme is inhibited to about 60–70% of its original activity by cross-linking with glutaraldehyde or in the presence of 1 M-$(NH_4)_2SO_4$, with its conformation stabilized as shown by the requirement for higher GuHCl concentrations to bring about both inactivation and unfolding. Low concentrations of GuHCl (0.2–0.4 M) activate the cross-linked and the $(NH_4)_2SO_4$-inhibited enzyme back to the level of the native enzyme. For the enzyme stabilized by $(NH_4)_2SO_4$ or by cross-linking with glutaraldehyde, inactivation occurs at a markedly lower GuHCl concentration than that required to bring about its first stage of unfolding. It is concluded that the active site of LDH is situated in a limited region relatively fragile in conformation as compared with the molecule as a whole. The GuHCl activation of LDH stabilized in $(NH_4)_2SO_4$ or by cross-linking with glutaraldehyde suggests that this fragility and consequently flexibility of the active site is required for its catalytic activity.

INTRODUCTION

Although the unfolding of proteins during the course of denaturation by chemical and physical factors has been extensively studied, relatively few attempts were made to correlate the loss of biological activities with the conformational changes of the proteins concerned (Creighton, 1984; Jaenicke, 1987). It has previously been shown in our laboratory that inactivation of several enzymes occurs before significant conformational changes of the molecules can be detected during denaturation by GuHCl, urea and heat (Yao *et al.*, 1984; Liu & Tsou, 1987; Liang *et al.*, 1990; Lin *et al.*, 1990). It is proposed that the active sites of these enzymes are situated in limited and relatively fragile regions whose conformational integrity is more sensitive to denaturation than the molecule as a whole. A slight perturbation either to the spatial geometry or to the correct dynamic properties of the active site responsible for catalysis destroys enzyme activity before the occurrence of any gross changes in the overall conformation of the molecule as a whole. Active-site fragility and consequently flexibility may well be required for the full expression of the catalytic power of the enzymes (Tsou, 1986, 1989).

The unfolding of LDH during denaturation by heat, acid, GuHCl and urea has been studied in detail, and it was reported that the inactivation of the enzyme parallels the dissociation of the tetrameric enzyme into the monomer and unfolding of the molecule as monitored by fluorescence-emission intensity and ellipticity changes (Appella & Markert, 1961; Brand *et al.*, 1962; Rudolph & Jaenicke, 1976). On the other hand, it is known that LDH can be stabilized either by cross-linking the subunits (Gottschalk & Jaenicke, 1987) or in the presence of high concentrations of $(NH_4)_2SO_4$ (Girg *et al.*, 1983). It would be of interest to see whether, for the enzyme stabilized under these conditions, the inactivation of the enzyme can be differentiated from unfolding of the molecule during denaturation by GuHCl. In the present study, the unfolding of LDH with increasing concentrations of GuHCl is shown to occur in two stages, and inactivation approximately coincides only with the first stage. For LDH stabilized by $(NH_4)_2SO_4$ or by cross-linking with glutaraldehyde, during denaturation with increasing GuHCl concentrations inactivation occurs significantly before measurable unfolding of the molecule as a whole, as with the other enzymes studied previously.

MATERIALS AND METHODS

Lactate dehydrogenase H_4 was purified from pig heart as described by Neilands (1955), and showed a single band during electrophoresis. GuHCl was a BRL product, and NADH was from BDH. $(NH_4)_2SO_4$ was an analytical-grade reagent from Beijing Chemicals and was recrystallized once before use. All other reagents were local products of analytical grade, used without further purification.

Cross-linking of the enzyme with glutaraldehyde was done in an enzyme solution (0.1 mg/ml) in 0.1 M-sodium phosphate buffer, pH 7.5, containing 1 mM-EDTA by rapidly mixing with a small volume of glutaraldehyde to a final concentration of 10 mg/ml at 20 °C. After incubation for 2 min, 7.6 mg of solid $NaBH_4$ was then added with rapid mixing to give a final molar ratio of $NaBH_4$/glutaraldehyde of 2. The reaction was allowed to proceed for 20 min to stabilize the cross-linkage and to remove excess glutaraldehyde by reduction. The pH of the solution was then adjusted with 1 M-H_3PO_4 to 7.5 and the resulting solution was thoroughly dialysed against the same buffer containing 1 mM-EDTA and 1 mM-dithiothreitol.

Enzyme concentration was determined spectrophotometrically at 280 nm with an absorption coefficient of 1.40 (1 cm) for a 0.1% solution (Jaenicke *et al.*, 1971). For the assay of enzyme activity, the oxidation of NADH by pyruvate was monitored at 340 nm in a spectrophotometer thermostatically maintained at 25 °C. The reaction mixture contained 0.1 M-sodium phosphate

Abbreviations used: GuHCl, guanidinium chloride; LDH, lactate dehydrogenase.
* To whom correspondence should be addressed.

buffer, pH 7.6, 1 mM-EDTA, 1 mM-dithiothreitol, 0.18 mM-NADH and 0.6 mM-pyruvate. For the measurement of enzyme activity denatured in GuHCl in the presence and absence of $(NH_4)_2SO_4$, the reaction mixture always contained the same concentrations of GuHCl and $(NH_4)_2SO_4$ during activity assay. The polymeric state of the enzyme in GuHCl was determined by cross-linking with glutaraldehyde, followed by SDS/PAGE (Laemmli, 1970).

For unfolding studies, the enzyme was first incubated with the required concentration of GuHCl at 25 °C for 24 h in sodium phosphate buffer, pH 7.5, to allow the reaction to reach completion, before fluorescence and c.d. measurements and activity assay. Without GuHCl, the enzyme is completely stable under identical conditions.

Fluorescence measurements were made with a Hitachi MPF-4 spectrofluorimeter at 25 °C with an exciting wavelength of 280 nm. C.d. was determined with a Jasco J 500A spectropolarimeter and a Jasco DP 500N data processor. The far-u.v. c.d. spectra were scanned from 200 to 250 nm at 25 °C. Changes in intrinsic fluorescence and in the ellipticity at 220 nm were taken to indicate protein unfolding. For the native and cross-linked enzymes, c.d. measurements were made in cuvettes with 2 mm path-length, but for the enzyme in the presence of $(NH_4)_2SO_4$ the enzyme solutions were diluted and measurements made in cuvettes of 10 mm path-length in order to minimize aggregation of the enzyme.

RESULTS

Activity and conformation changes of LDH during denaturation with GuHCl

Changes in the emission intensity at 340 nm and the position of the emission maximum of the intrinsic fluorescence, ellipticity at 220 nm and activity of the H_4 isozyme of LDH after denaturation for 24 h in GuHCl solutions of different concentrations are shown in Fig. 1. The enzyme is stable in GuHCl up to about 0.4 M, but loses its activity sharply with increasing concentrations of the denaturant and is completely inactivated in 1 M-GuHCl. The unfolding of the molecule occurs in two stages with increasing concentrations of GuHCl. The initial stage of fluorescence and c.d. changes takes place at GuHCl concentrations only slightly higher than that required for inactivation of the enzyme involving a decrease in emission intensity, a slight blue-shift in emission maximum and a decrease in the amplitude of negative ellipticity. After the inactivation of the enzyme has reached completion, further decreases in emission intensity and the amplitude of negative ellipticity occur at GuHCl concentrations higher than 2 M, showing further unfolding of the molecule, as is also evident from a marked red-shift of the emission maximum of its intrinsic fluorescence at the second stage.

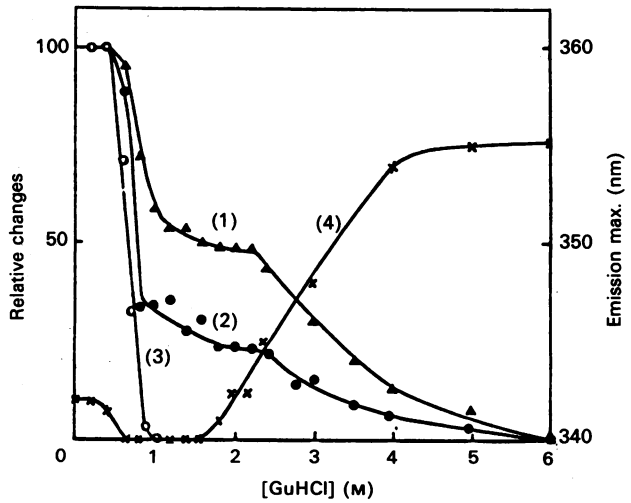

Fig. 1. Activity and conformation changes of LDH during denaturation with GuHCl

Left-hand ordinate indicates relative changes in ellipticity at 220 nm (1), fluorescence emission intensity at 340 nm (2) and enzyme activity (3). The right-hand ordinate shows the shift in emission maximum (4). The enzyme (0.36 μM) was incubated for 24 h in phosphate buffer, pH 7.5, containing the required concentration of GuHCl at 25 °C for fluorescence and c.d. determinations. It was diluted to 1.4 nM in the same solution for activity assay.

For the enzyme completely inactivated in 1 M-GuHCl, analysis by cross-linking with glutaraldehyde and SDS/PAGE after 100-fold dilution shows that the inactive and partly denatured enzyme is in the monomeric form, as shown in Fig. 2. This is in general agreement with the result of Jaenicke *et al.* (1981). It is known that under similar conditions native LDH exists exclusively as the tetramer. It is clear that the first stage of denaturation involves inactivation, dissociation to the monomer and partial unfolding of the monomeric form, whereas further unfolding of the monomer occurs at the second stage until complete disappearance of ordered secondary structure in 6 M-GuHCl, as far as can be ascertained by c.d. measurements.

Activity and conformation changes of cross-linked LDH during denaturation with GuHCl

LDH can be stabilized by cross-linking the subunits with glutaraldehyde (Gottschalk & Jaenicke, 1987), and the extent of cross-linking depends on the concentrations of both the enzyme and the cross-linking reagent. Under the conditions employed in this investigation, the enzyme is almost entirely cross-linked, and no monomeric or dimeric forms can be detected, as shown by SDS/PAGE (Fig. 2). It has an activity about 60–70% of that of the native enzyme. Fig. 3 shows the comparison of the unfolding and inactivation of the cross-linked enzyme in different concentrations of GuHCl. At low GuHCl concentrations (0.2–0.4 M), the cross-linked enzyme is activated to a level comparable with that of the native enzyme, and this is accompanied by an increase in emission intensity of its intrinsic fluorescence, which, however, takes place at slightly higher GuHCl concentrations. The cross-linked enzyme is more stable to the denaturing action of GuHCl than is unmodified LDH, as higher concentrations of the denaturant are required for both inactivation and the initial stages of fluorescence and ellipticity changes. Furthermore, markedly higher concentrations of the denaturant are now required for the initial stage of unfolding, as indicated by both fluorescence and c.d. changes, including the slight blue-shift of the emission maximum, than that required to bring about complete inactivation of the enzyme. It is clear that, when the enzyme is stabilized by cross-linking, inactivation takes place before marked conformation change can be observed. It should be noted that with increasing GuHCl concentration a region of relatively little change at about 2 M is discernible by c.d. measurements, but the initial stage merges into the second continuously with fluorescence-emission intensity measurements. The fluorescence maximum shifts to the red after inactivation of LDH has reached completion.

For the unmodified enzyme, dissociation takes place at 1 M-GuHCl, accompanied by both partial unfolding and complete

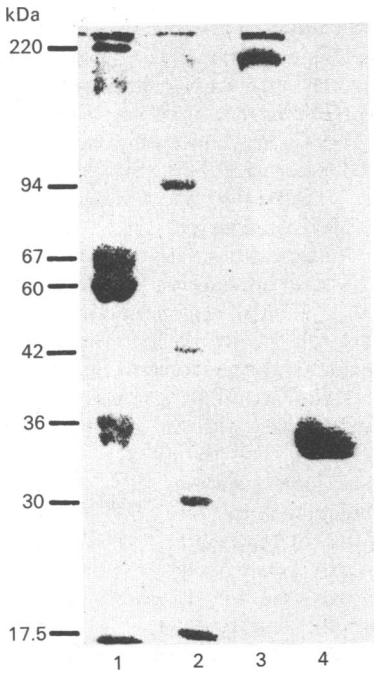

Fig. 2. SDS/PAGE of cross-linked LDH

Experimental conditions were as described in the Materials and methods section. Lane 1, Molecular-mass standard proteins: ferritin (220 kDa), BSA (67 kDa), catalase (60 kDa), LDH (36 kDa), tobacco-mosaic-virus (TMV) coat protein (17.5 kDa); lane 2, molecular-mass standard proteins: phosphorylase *b* (94 kDa), rabbit muscle actin (42 kDa), carbonic anhydrase (30 kDa), TMV coat protein (17.5 kDa, as in lane 1); lane 3, cross-linked LDH; lane 4, LDH denatured in 1 M-GuHCl for 24 h before cross-linking with glutaraldehyde. Note also the position for native LDH in lane 1.

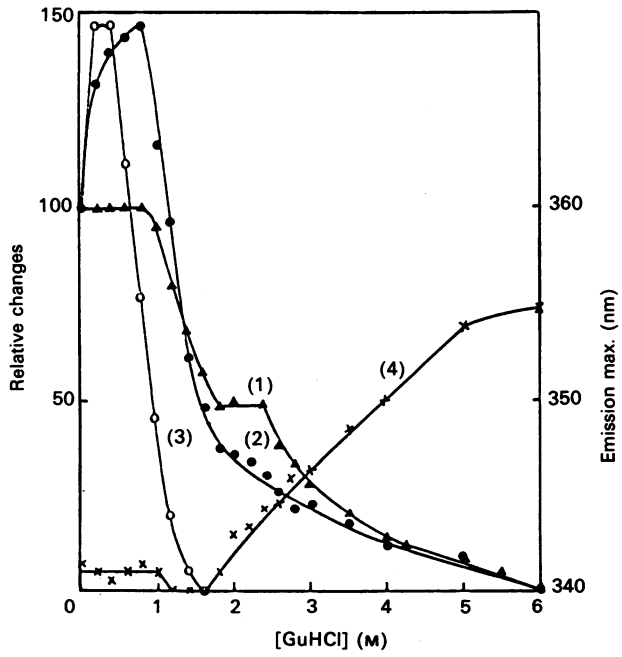

Fig. 3. Activity and conformation changes of cross-linked LDH during denaturation with GuHCl

Experimental conditions and numbering of the curves are the same as for Fig. 1, except that the cross-linked enzyme at the same concentration was used.

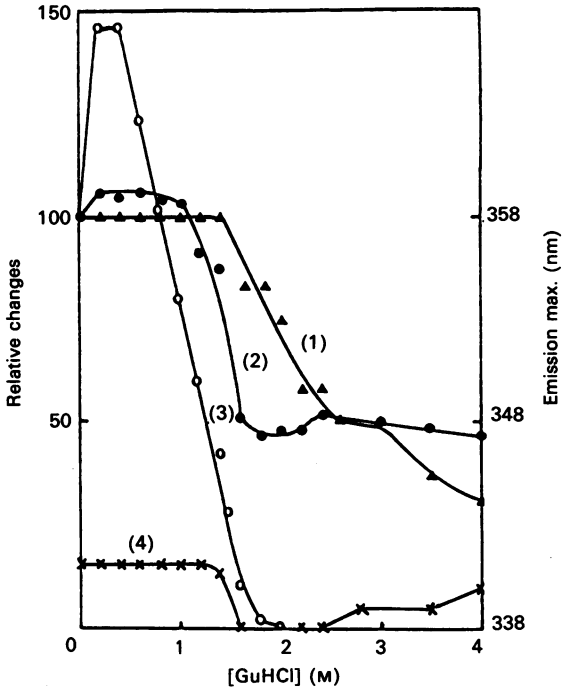

Fig. 4. Activity and conformation changes of LDH in $(NH_4)_2SO_4$ during denaturation with GuHCl

Experimental conditions and numbering of the curves are the same as for Fig. 1, except that the initial enzyme concentration was 0.14 μM, and 1 M-$(NH_4)_2SO_4$ was also present during both the incubation with GuHCl and the subsequent measurements.

inactivation. The association of the subunit to form the tetramer would require that the subunits are in a native folded state. Although cross-linking of the enzyme covalently prevents its dissociation, the above results show that, when the enzyme is already largely inactivated, the molecule has not unfolded to a marked extent, as measured by fluorescence and ellipticity changes. Similarly, it has been previously shown that the inactivation of both creatine kinase and D-glyceraldehyde-3-phosphate dehydrogenase in GuHCl takes place before significant dissociation of the respective oligomeric molecules (Liu *et al.*, 1989; Liang *et al.*, 1990).

Activity and conformation changes of LDH in $(NH_4)_2SO_4$ during denaturation with GuHCl

$(NH_4)_2SO_4$ is known as a structure-stabilizing salt, and LDH is stabilized in its presence in high concentrations (Girg *et al.*, 1983), suggesting a more rigid structure of the enzyme. The enzyme is inhibited by $(NH_4)_2SO_4$ at 1 M to an extent of about 30–40%. Like the cross-linked enzyme, it is activated back to the level of the native enzyme when 0.2–0.4 M-GuHCl is also present (Fig. 4). However, this activation is not accompanied by an increase in the emission intensity of its intrinsic fluorescence, as is the case for the cross-linked enzyme. The stabilizing effect of $(NH_4)_2SO_4$ is shown by the facts that much higher concentrations of GuHCl are required for the inactivation and unfolding of LDH in 1 M-$(NH_4)_2SO_4$ than in its absence, as is evident from a comparison of the results given in Figs. 1 and 4. Complete inactivation now occurs at about 1.8 M-GuHCl, and no second stage of unfolding can be discerned up to 4 M-GuHCl. In 1 M-$(NH_4)_2SO_4$, the fluorescence emission maximum shifts slightly to the blue, together with the inactivation of the enzyme, but does not show the marked red-shift evident in absence of the salt, demonstrating that the enzyme has considerable ordered struc-

ture even in 4 M-GuHCl because of the stabilizing effect of the salt. It is also clear from Fig. 4 that, with increasing concentrations of GuHCl, inactivation of the $(NH_4)_2SO_4$-stabilized enzyme markedly precedes its unfolding, as indicated by fluorescence and c.d. measurements.

DISCUSSION

It has been proposed that the unfolding of proteins in denaturants can be adequately described by a simple-equilibrium two-state model (Tanford, 1968; Privalov & Khechinashvili, 1974) as follows:

$$N \rightleftharpoons U$$

where N is the native folded form and U is the unfolded form of the protein, implying that no relatively stable folding intermediates exist and the unfolding is an all-or-none process. Evidence in support of the two-state model involving conformational changes measured by different physical parameters at different denaturant concentrations shows the same extents of change under similar conditions (Nojima *et al.*, 1977), and stable intermediates during either unfolding or refolding are rarely observed (Kim & Baldwin, 1982). However, more recently it has been repeatedly shown that such intermediates do exist during both unfolding (Tsou, 1986) and refolding (Biringer & Fink, 1988).

The unfolding of muscle LDH apparently occurs in two stages with increasing concentrations of GuHCl, with the first stage approximately coinciding with its inactivation. Similar results have been reported for muscle D-glyceraldehyde-3-phosphate dehydrogenase (Xie & Tsou, 1987; Ju & Tsou, 1988). The presence of a plateau of little change suggests the presence of a relatively stable intermediate unfolded state for these enzymes. This intermediate stage for LDH is completely inactive and monomeric, but appears to be only partly unfolded, as far as can be ascertained by fluorescence and c.d. measurements. Further unfolding of the molecule occurs at higher GuHCl concentrations.

Among the vast amount of literature on protein unfolding, only a limited number of authors have attempted to correlate conformation and activity changes during the course of denaturation. Although some authors have observed that inactivation occurs before measurable conformational changes (Nieto & Ayala, 1977; Wolfgang & Hess, 1981; Vecchio *et al.*, 1987), others reported parallel changes in both parameters (Busby *et al.*, 1981; Morl *et al.*, 1981; Withka *et al.*, 1987; Johnson & Price, 1987). However, closer examinations of some papers reporting parallel decrease in activity with conformation changes, as measured by the usual physico-chemical methods, sometimes reveal that inactivation actually occurred before the unfolding of the molecule (Tsou, 1989). For those reports that parallel changes in activity and in conformation were indeed observed, the following have still to be considered. (*a*) The inactivation was usually carried out by the conventional method of incubating the enzyme with the denaturant for a definite time interval and then taking samples and diluting into the reaction mixture for activity assay. As the denaturant is usually diluted many-fold with the enzyme, some re-folding and hence re-activation may have occurred. (*b*) Even with the precaution, as taken by some authors, of using as small a dilution factor as possible and diluting the enzyme into an assay mixture containing the same concentration of the denaturant, the presence of substrates in the assay mixture can sometimes bring about some re-activation, especially for the partially inactivated enzyme at the initial stages of unfolding.

Evidently, either or both of the above can bring about an underestimation of the extent of inactivation during the course of denaturation. For LDH, even though the inactivation coincides approximately with first-stage conformational changes at low concentrations of GuHCl, further unfolding of the molecule has been observed at higher GuHCl concentrations. Moreover, with the enzyme stabilized either with $(NH_4)_2SO_4$ or by cross-linking with glutaraldehyde, inactivation takes place before the first stage of unfolding, as far as can be measured by fluorescence and c.d. studies. All the above substantiate the previous suggestion that like the other enzymes previously studied, the active site of LDH is situated in a limited region of the enzyme whose conformation is held together by weaker forces and is hence more fragile to perturbation by denaturants than the molecule as a whole (Tsou, 1989). Similar results have been obtained for 20-hydroxysteroid dehydrogenase with and without stabilizing salts (Vecchio *et al.*, 1987).

The activation at low concentrations of GuHCl of the enzyme stabilized and partly inhibited by $(NH_4)_2SO_4$ or by cross-linking back to the level of the native enzyme is remarkable, a relatively fragile and consequently flexible active site has been envisaged which may well be required for the catalytic activity of the enzyme. The partial inhibition by $(NH_4)_2SO_4$ and cross-linking by glutaraldehyde could be due to their stabilizing effect on the conformation of the enzyme resulting in a more rigid active site. Consequently, the activation by low concentrations of GuHCl could then be due to the restoration to the stabilized enzyme of the flexibility at the active site required for the full expression of its activity.

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