Stepwise Inactivation of *Escherichia coli* Aspartokinase–Homoserine Dehydrogenase I†

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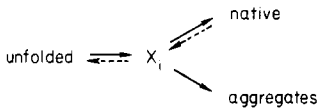
ABSTRACT: In the range of guanidine hydrochloride concentrations from 0.2 to 1.2 M, aspartokinase–homoserine dehydrogenase I loses its enzymatic properties, both kinase and dehydrogenase activities and their allosteric inhibition by L-threonine. Ligands which stabilize the tetrameric native structure protect the enzyme against inactivation. Under some conditions, all the functional properties do not disappear at the same rate: an intermediate species possessing only the kinase activity can be detected. Several arguments suggest that this partly active intermediate has a monomeric structure. These results show that deactivation of aspartokinase–homoserine dehydrogenase I is a stepwise process, compatible with the reverse of the previously described reactivation [Garel, J.-R., & Dautry-Varsat, A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3379–3383]. The same measurements performed with a monofunctional fragment carrying the dehydrogenase activity show that the loss of dehydrogenase activity is the same whether or not the polypeptide chain is intact or lacks the kinase region; this finding suggests that the protein is composed of independent regions. The influence of protein aggregation in studying unfolding–refolding of oligomeric enzymes is also discussed.

In many cases, the native structure of an oligomeric protein has been reconstituted from its unfolded and separated chains (Perrin & Monod, 1963; Groha et al., 1978; Jaenicke et al., 1979; Zettlmeissl et al., 1982). However, the yield with which the native state is recovered depends strongly on experimental conditions, and particularly on protein concentration because of aggregation. Aggregation is a quasi-irreversible reaction in which part of the protein comes out of solution before it has reached the native conformation. The yield of reconstitution will depend on the relative rates of two competing reactions in which partially folded intermediates (X_i) can either aggregate or directly refold to the native form (Jaenicke & Rudolph, 1980):



Aggregation shows some specificity: the yield of renaturation of a given protein is not affected by the presence of other proteins (Cook & Koshland, 1969; London et al., 1974; Jaenicke et al., 1981). However, despite the fact that it is probably related to some specific partial folding, aggregation has been (and will be) considered as a major complication in studying the folding and self-assembly of oligomeric proteins (Kim & Baldwin, 1982). Since the competition between renaturation and aggregation is essentially kinetic, conditions which minimize aggregation are those which decrease its rate and increase that of renaturation; the conditions which are most generally used involve low protein concentrations (which slow down aggregation) and strongly native solvents (which accelerate the formation of the native state) (Jaenicke, 1974; Teipel & Koshland, 1971). Therefore, refolding of an oligomeric protein is almost always studied under "far from equilibrium" conditions. In fact, this perturbing influence of aggregation is such that there are very few reports on the equilibrium between unfolded monomers and native oligomers, whereas there are many examples of quantitative renaturation of unfolded monomers into native oligomers (Jaenicke, 1982).

The refolding pathway of aspartokinase–homoserine dehydrogenase I (AK–HDH I)[1] from *Escherichia coli* has been previously studied under such irreversible conditions, by measuring the kinetics of regain of the functional properties (Garel & Dautry-Varsat, 1980a). AK–HDH I is a bifunctional enzyme, and its two activities, kinase and dehydrogenase, are allosterically inhibited by the same effector, L-threonine (Patte et al., 1966; Truffa-Bachi et al., 1974). The reappearance of the various catalytic and regulatory properties corresponds to three different time courses depending on the property, showing that self-assembly of AK–HDH I involves at least three steps; a kinetic analysis of these results leads to the tentative assignment of these steps as (a) a folding reaction of the monomer, (b) the association of two folded monomers into a dimer, and (c) the association of two dimers into the native tetramer (Garel & Dautry-Varsat, 1980a,b). Because of the final nonequilibrium conditions, finding a sequential pathway of refolding of AK–HDH I gives information only on the relative rates of the various reactions and not on the relative stabilities of the intermediate species present during renaturation. For instance, a monomeric intermediate will be observed if its folding is fast as compared to its subsequent dimerization, even if this monomeric species is much less stable than the dimer. In order to assess the reversibility of the folding pathway of AK–HDH I, the behavior of the protein has been studied in the presence of moderate concentrations of the denaturant guanidine hydrochloride (Gdn·HCl), i.e., under conditions where the native tetramer is no longer the most stable species.

Materials and Methods

Materials

Inorganic potassium salts (p.a.) were obtained from Merck (Darmstadt, West Germany); guanidine hydrochloride (ultrapure) was from Schwarz/Mann (New York, NY). Dithiothreitol, NADH, NADPH, ATP, and phosphoenolpyruvate

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[1] Abbreviations: AK–HDH I, aspartokinase–homoserine dehydrogenase I (EC 2.7.2.4 and EC 1.1.1.3); Gdn·HCl, guanidine hydrochloride; DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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were from Sigma, L-aspartic acid (A grade) was from Calbiochem, EDTA (dipotassium salt) was from Fluka, and DL-threonine was from AEC (Commentry, France). Aspartate semialdehyde was prepared by ozonolysis of DL-allylglycine (Black & Wright, 1955). The auxiliary enzymes (lactic dehydrogenase and pyruvate kinase) used in the coupled assay of aspartokinase activity (Wampler & Westhead, 1968) were purchased from Sigma. AK–HDH I was purified from *E. coli* K12 according to Janin & Cohen (1969). The dehydrogenase fragment was obtained from native AK–HDH I by limited proteolysis with Pronase (Fazel et al., 1983). Both AK–HDH I and its fragment were stored as an ammonium sulfate precipitate (50% saturation). Before use, the proteins were repeatedly dialyzed against a buffer containing 0.1 M potassium phosphate, 0.5 M potassium chloride, 10 mM dithiothreitol, 1 mM EDTA, and 2 mM L-threonine at pH 7.2.

Methods

Enzymatic Assays. Aspartokinase activity was determined by using the coupled assay of Wampler & Westhead (1968) in 0.35 M KCl and 0.17 M Tris-HCl at pH 7.4. Homoserine dehydrogenase activity was directly measured by reduction of aspartate semialdehyde in 1.0 M KCl and 0.1 M potassium phosphate at pH 7.2 (Truffa-Bachi & Cohen, 1970). In both cases, the inhibition by L-threonine was determined for an effector concentration of 2 mM. The changes of absorbance at 340 nm in both measurements were followed with a Gilford 240 spectrophotometer.

Deactivation Studies. For deactivation studies, AK–HDH I or its dehydrogenase fragment was incubated in a medium containing 0.1 M potassium phosphate, 0.5 M KCl, and 10 mM dithiothreitol at pH 7.2 and a given concentration of Gdn·HCl. The amount of activity remaining after a given time was measured after rapidly transferring the protein into the standard assay mixture, i.e., after diluting out the Gdn·HCl. The residual Gdn·HCl concentration was always less than 50 mM and was checked not to interfere with activity assays. Dilution of the protein and measurement of its activity were always achieved within 2 min; these 2 min then correspond to the "dead time" of the method. This time is much shorter than the half-lives for the reappearance of the two activities upon refolding AK–HDH I from its unfolded chains in the same conditions (Garel & Dautry-Varsat, 1980a,b); also, the absorbance vs. time profiles obtained in activity measurements are the same as those obtained with the native enzyme, which should not be the case if significant reactivation occurred during the assay.

Results

Figure 1 gives the enzymatic activity, kinase or dehydrogenase, which remains after incubation of AK–HDH I for a given time in various Gdn·HCl concentrations. The enzyme is stable for 24 h in Gdn·HCl up to 0.2 M: both activities are fully preserved, as well as their inhibition by L-threonine. Above 0.2 M Gdn·HCl, AK–HDH I is progressively inactivated, to an extent which increases with the incubation time and the Gdn·HCl concentration. This deactivation of AK–HDH I is irreversible because it is coupled to the precipitation of the protein; indeed, the samples become more and more turbid, indicating the extensive formation of insoluble aggregates. Gdn·HCl apparently induces a change in the conformation of AK–HDH I which alters its solubility. Figure 1 also shows that deactivation is about the same for the two activities. These two activities are affected at the same rate, at least under some conditions: in 0.55 M Gdn·HCl, the time courses of irreversible inactivation of the kinase and

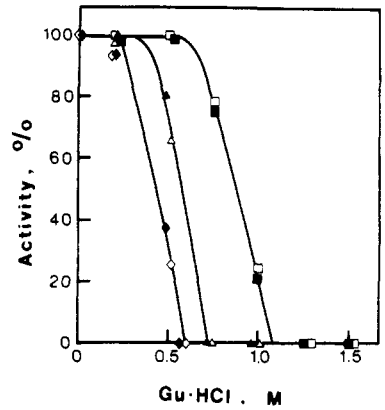

FIGURE 1: Residual catalytic activities of AK–HDH I after (□, ■) 10 min, (△, ▲) 3 h, and (◇, ◆) 24 h of incubation in the presence of Gdn·HCl and potassium phosphate buffer (0.1 M), pH 7.2, plus 0.5 M KCl and 10 mM DTT, 27 °C. Protein concentration was 17.5 μg/mL (0.2 μM). Closed symbols, kinase activity; open symbols, dehydrogenase activity.

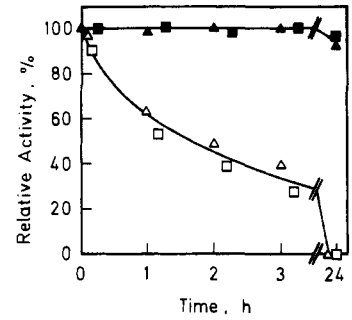

FIGURE 2: Time course of the loss of (△) kinase and (□) dehydrogenase activities of AK–HDH I in the presence of 0.55 M Gdn·HCl. Conditions of incubation as in Figure 1. The filled symbols correspond to incubation in the presence of 0.55 M Gdn·HCl and 2 mM L-threonine.

dehydrogenase functions are indeed very similar (Figure 2). Both activities are protected by L-threonine (Figure 2), and no precipitation of AK–HDH I is observed upon incubation in Gdn·HCl and in the presence of the allosteric effector; L-threonine prevents the conformational change induced by Gdn·HCl from taking place. Besides the inhibition of activity, the binding of L-threonine to AK–HDH I has a structural influence: it protects the tetrameric enzyme from dissociation into dimeric species (Truffa-Bachi et al., 1968; Mackall & Neet, 1973; Cohen & Dautry-Varsat, 1980). The correlation between protecting AK-HDH I from irreversible inactivation by Gdn·HCl and stabilizing the tetramer from dissociation in the absence of denaturant can be extended to ligands of AK–HDH I other than L-threonine. Aspartate or NADPH by themselves slow down the inactivation (Table I) and stabilize weakly the tetrameric structure; both these effects are amplified when the two ligands are used together: an almost complete maintenance of the activity (Table I) and little dissociation into dimeric species (unpublished results). The liganded forms of AK–HDH I are more stable than the free enzyme toward dissociation into dimers and inactivation by Gdn·HCl, independently of the nature, substrate or effector, of the ligand. This suggests that the first (or one of the first) step(s) involved in the deactivation by Gdn·HCl could be a dissociation of AK–HDH I; dissociated species would indeed be expected to be less soluble than the native enzyme and therefore to show a more pronounced trend toward aggregation and precipitation.

Figure 2 shows that the kinase and dehydrogenase activities are lost at about the same rate upon incubation at a given

Table I: Protection against Inactivation of AK-HDH I by Various Ligands

ligand added	residual activity (%)[a]	
	kinase	dehydrogenase
none	50	25
0.25 mM NADPH	80	50
10 mM aspartate	85	50
0.25 mM NADPH plus 10 mM aspartate	90	65
2 mM L-threonine	95	90

[a] Residual activities were measured after 3 h of incubation in 0.65 M Gdn·HCl at 27 °C and potassium phosphate buffer (0.1 M), pH 7.2, plus 0.5 M KCl and 10 mM DTT. The protein concentration was 17.5 μg/mL (0.2 μM).

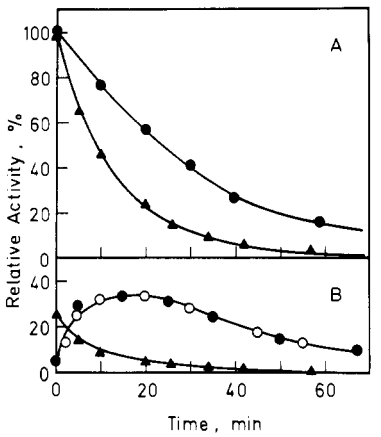

FIGURE 3: Kinetics of the loss of enzymatic functions of AK-HDH I in the presence of 0.75 M Gdn·HCl. Conditions of incubation as in Figure 1. (A) Disappearance of (●) kinase activity and (▲) dehydrogenase activity. (B) Residual amount of (●) kinase activity and (▲) dehydrogenase activity measured when 2 mM L-threonine is present in the enzymatic assay. (○) gives the difference between the kinase activity and the dehydrogenase activity as calculated from the curves shown in (A).

Gdn·HCl concentration. This is not true at all Gdn·HCl concentrations. Indeed, raising slightly the Gdn·HCl concentration from 0.55 to 0.75 M causes the dehydrogenase activity to disappear faster than the kinase activity (Figure 3A). The inhibition by L-threonine of the two residual activities is also different; the dehydrogenase activity is always inhibited to the same extent, about 70%, throughout the whole kinetics of inactivation. The kinase activity, however, becomes more and more resistant to threonine inhibition as deactivation proceeds: for instance, after about 30 min of inactivation, the kinase activity has decreased to 40% of its initial value, but it is inhibited to only 30% as compared to 95% for the original enzyme (Figure 3). This shows that the sensitivity to threonine of the kinase activity is lost faster than the activity itself in these conditions; finding that these two properties follow different kinetics shows that the reaction involves at least one intermediate species (Tanford, 1970). This intermediate would still possess the kinase activity but would have already lost the inhibition of this activity by L-threonine. A convenient way to monitor this intermediate is therefore to measure the kinase activity in the presence of 2 mM L-threonine, i.e., a concentration which almost completely inhibits the native enzyme. Figure 3B shows that the "threonine-resistant" kinase activity increases with time to reach a maximum level and then decreases, as expected from the formation and subsequent decomposition of a transient intermediate. This is not the case when the dehydrogenase activity is assayed in the presence of a saturating concentration of L-threonine: both the threonine-resistant and the total dehydrogenase activities disappear monotonously and at the same rate (Figure 3). This rate is the same as that of disappearance of the threonine sensitivity of the kinase activity, which suggests that the intermediate species possesses only the kinase activity and has lost all the other functional properties of AK-HDH I; indeed, the relative amount of a species with an active kinase and an inactive dehydrogenase, which is given by the difference between the two curves of Figure 3A, is in good agreement with that of the intermediate species, as determined above by comparing the time courses of the kinase activity and its inhibition by L-threonine (Figure 3B). This agreement indicates that the same intermediate species is characterized by these two independent procedures and hence that this species has kept only its kinase activity.

Similar experiments were also performed on a dimeric fragment of AK-HDH I obtained by limited proteolysis and which possesses only the dehydrogenase activity (Véron et al., 1972; Fazel et al., 1983). Incubation in Gdn·HCl inactivates this fragment as in the case of the entire protein; L-threonine does not inhibit the activity of this fragment (Véron et al., 1972; Fazel et al., 1983) and has no influence on the rate of inactivation at a given Gdn·HCl concentration (not shown). It can be pointed out that the inactivations by Gdn·HCl of the dehydrogenase activity of the entire AK-HDH I (Figure 1) and of its proteolytic fragment are very similar, although the former is tetrameric and the latter only a dimer of shortened chains; this suggests that the structural differences between these two protein species have only a minor influence on the maintenance of the dehydrogenase activity.

Discussion

Under a limited set of conditions, the functional properties of AK-HDH I do not disappear at the same rate upon incubation of the enzyme in moderate Gdn·HCl concentrations. The results are consistent with a three-state mechanism, in which one intermediate species would be transiently present between a fully functional protein and an inactive material (Figure 3). This intermediate is no longer inhibited by L-threonine and no longer has a dehydrogenase activity but has maintained a kinase activity. This intermediate species can be tentatively identified as a folded monomer on the basis of the following arguments: (i) the effects of various ligands and especially L-threonine suggest that dissociation is one of the first steps of deactivation (see above); (ii) dimeric derivatives of AK-HDH I are still endowed with both kinase and dehydrogenase activities, each of them sensitive to L-threonine (unpublished results); thus, a tetramer to dimer dissociation may not involve a significant change in enzymatic properties; (iii) in more concentrated Gdn·HCl solutions, ca. 2–3 M, the stable state of AK-HDH I is monomeric, folded, and soluble [see Müller & Garel (1984)]; (iv) an intermediate with exactly the same functional properties has been previously characterized in refolding as a folded monomeric species (Garel & Dautry-Varsat, 1980a).

This last argument strongly suggests that at least one intermediate, with the properties given above, is present on both the unfolding and refolding pathways of AK-HDH I. Then, despite the fact that both unfolding and refolding have been studied under far from equilibrium conditions, finding a common intermediate indicates that they proceed through the same or similar steps, i.e., that they are the reverse of each other.

The partly active monomeric intermediate can be detected only under some conditions, where its relative rates of formation and disappearance are such that it accumulates tran-

siently (Figure 3B). These rates have such a strong dependence on Gdn·HCl concentration that this intermediate does not accumulate outside a narrow range of Gdn·HCl concentrations around 0.75 M: at 0.55 M, it is not seen or barely seen (Figure 2), and at 0.95 M, deactivation is too fast to measure. However, the direct observation of this intermediate is sufficient to establish that deactivation of AK–HDH I is not an all or none process.

The intermediate species is apparently monomeric and still (at least partly) folded (see above). This indicates that incubation in moderate Gdn·HCl concentration destroys the interactions between subunits without affecting too much the interactions within a subunit. A similar conclusion is more firmly established in the following paper (Müller & Garel, 1984).

There is very little difference between the deactivation of the dehydrogenase activity in either the entire tetrameric AK–HDH I or the dimeric proteolytic fragment. The stability of the region responsible for this activity does not apparently depend on the presence of (a) a tetrameric structure and (b) the rest of the polypeptide chain. This region was previously found to be an independent folding unit (Dautry-Varsat & Garel, 1981); it seems also to be an independent unfolding unit.

The same method, activity measurements, has been quite successful in determining a detailed pathway for the folding of AK–HDH I (Dautry-Varsat & Garel, 1981), whereas here it allows one to reach only limited conclusions. This contrast is due to the assymmetric influence of protein aggregation on unfolding and refolding; indeed, the marked decrease in solubility upon going from a native to a (partly) denatured state results in an unavoidable perturbation of unfolding studies. Further investigation of the various successive steps involved in the disruption of the native conformation of AK–HDH I requires other methods, an example of which is given in the following paper (Müller & Garel, 1984).

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Registry No. Gdn·HCl, 50-01-1; NADPH, 53-57-6; L-aspartic acid, 56-84-8; L-threonine, 72-19-5.

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