Folding of Aspartokinase–Homoserine Dehydrogenase I Is Dominated by Tertiary Interactions†

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ABSTRACT: In the presence of guanidine hydrochloride concentrations above 2 M, aspartokinase–homoserine dehydrogenase I remains sufficiently soluble so that the fluorescence and circular dichroism of the protein can be measured. Both parameters show that, up to 3 M guanidine hydrochloride, the protein exists in a stable folded state which possesses a large amount of secondary structure and buried tryptophan residues. This intermediate species is probably monomeric; it is reversibly unfolded by guanidine hydrochloride concentrations between 3 and 4 M. This folded species is formed rapidly from unfolded protein when the denaturant is diluted out, and this rapid folding step precedes all the reactivation steps described previously. The existence of a stable monomeric and folded intermediate indicates that the tertiary interactions have a major contribution to the stability of the native structure of aspartokinase–homoserine dehydrogenase I. Similar measurements were performed on two complementary nonoverlapping fragments: a kinase fragment corresponding to the N-terminal third and a dehydrogenase fragment corresponding to the C-terminal two-thirds of the polypeptide chain. Both fragments exist in a stable folded state up to 2.5 M guanidine hydrochloride. Both fragments show cooperative unfolding transitions between 2.5 and 4 M denaturant. The stability of the folded state of a given region is about the same in an isolated fragment and in the entire chain of aspartokinase–homoserine dehydrogenase I: indeed, an equimolar mixture of these two fragments and the intact chain would give about the same results. This indicates that folding of the kinase and dehydrogenase regions occurs independently with a single subunit of the entire protein.

Aspartokinase–homoserine dehydrogenase I (AK–HDH I)[1] from *Escherichia coli* is a bifunctional protein composed of four identical polypeptide chains (M_r 89 000) each carrying the sites involved in both activities; the kinase activity corresponds to the amino-terminal part of each chain and the dehydrogenase activity to the carboxy-terminal part (Véron et al., 1972; Thèze & Saint-Girons, 1974). The native state of AK–HDH I has a tetrameric structure, but two different lines of evidence suggest that functional (and therefore folded) species can exist as monomers in the absence of any subunit interactions: (a) limited proteolysis of native AK–HDH I yields monofunctional fragments which are monomeric and active (Fazel et al., 1983); (b) the kinetics of deactivation and reactivation of AK–HDH I are compatible with the existence of a monomeric intermediate possessing the kinase activity (Garel & Dautry-Varsat, 1980a; Müller & Garel, 1984).

These results suggest that the polypeptide chain of AK–HDH I, or an appropriate segment of it, can take or remain in a conformation which is close to that in the native protein, without being stabilized by interactions between different chains; such a folded conformation is maintained only by interactions of the chain with itself. It seems then as if the tertiary interactions are by themselves sufficient to drive the folding of an isolated chain of AK–HDH I (or one of its fragments) into a quasi-native conformation. The quaternary interactions would have a minor role in the folding process, albeit they would obviously be important for the expression of the catalytic and/or regulatory properties of the final folded state; this conclusion is consistent with the mechanism proposed earlier for the self-assembly of AK–HDH I (Dautry-Varsat & Garel, 1981).

In the case of the entire AK–HDH I chain, folded monomers have only been observed as transient intermediates in deactivation or reactivation (Garel & Dautry-Varsat, 1980a; Müller & Garel, 1984); also, the lability of the monomeric active fragments obtained by proteolysis indicates that they could only correspond to metastable species (Fazel et al., 1983). In addition, only activity measurements have been used to establish the formation of a folded structure in a given segment of the chain; it is obvious that such a functional definition of a folded state is strongly biased since large segments of a polypeptide chain can take a stable, specific, and highly organized conformation without being active. Therefore, although it is clear that the tertiary interactions dominate the folding of AK–HDH I, it is difficult to estimate the stability of the folded monomeric species from previous data.

In the present work, the formation of folded structures in the entire chain of AK–HDH I or a part of it is monitored by physical parameters: circular dichroism and fluorescence. Circular dichroism (CD) is related to the presence of secondary structure in the polypeptide backbone (Adler et al., 1973), whereas fluorescence monitors the local environment surrounding the aromatic side chains, mostly tryptophan residues (Brand & Witholt, 1967).

Materials and Methods

Materials. Unless otherwise stated, all materials used in this study are the same as those given in the preceding paper (Müller & Garel, 1984). Buffer solutions were prepared with quartz bidistilled water in the case of CD measurements; water which had been pyrolyzed after deionization was utilized in fluorescence studies: the traces of fluorescent organic molecules present in water are destroyed more satisfactorily by this high-temperature treatment than by any other method. The

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[1] Abbreviations: AK–HDH I, aspartokinase–homoserine dehydrogenase I (EC 2.7.2.4 and EC 1.1.1.3); CD, circular dichroism; Gdn·HCl, guanidine hydrochloride; DTT, 1,4-dithiothreitol.

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sensitivity of fluorescence measurements on dilute protein solutions depends critically on the quality of the water. The kinase fragment, as the dehydrogenase fragment, was obtained by limited proteolysis of AK–HDH I with Pronase (Fazel et al., 1983).

Methods. In order to circumvent artifacts due to solvent impurity, special care was taken in the preparation of solutions: (i) highly purified water was used throughout (see above); (ii) buffer solutions were prepared freshly every day. Gdn·HCl concentrations were measured by refractometry (Kielley & Harrington, 1960). All incubations and measurements were performed at 27 °C. Protein fluorescence was followed with a JOBIN YVON JY3 spectrofluorometer; CD measurements made use of a Jasco 500-A circular dichroism spectropolarimeter equipped with a Jasco DP-500 N data processor. All spectroscopic data were corrected with respect to buffer.

Results and Discussion

One of the major complications encountered in the study of the unfolding–refolding process of oligomeric proteins is the irreversible aggregation of partially folded species (Jaenicke, 1982); in the case of AK–HDH I, quantitative renaturation can only be achieved if the protein concentration is maintained at a low value (Garel & Dautry-Varsat, 1980a). The preceding paper (Müller & Garel, 1984) shows that a marked change in the solubility of AK–HDH I occurs when the Gdn·HCl concentration is raised above 0.2 M, probably as a result of the protein dissociation. Increasing the Gdn·HCl concentration above 0.2 M accelerates this aggregation [see Figure 1 of Müller & Garel (1984)] because partial unfolding is favored. However, at 2 M Gdn·HCl and above, the protein remains soluble, and its fluorescence and CD can then be measured in the presence of 2–6 M denaturant. Indeed, light-scattering studies (not shown) were used to check that aggregation was negligible in all the experiments described later in this paper. This absence of aggregation at higher Gdn·HCl concentrations is probably related to the fact that Gdn·HCl is a good solvent for (partially) unfolded species, hence its denaturing power (Tanford, 1968).

AK–HDH I Retains a Stable Folded Structure in 3 M Gdn·HCl. The fluorescence emission spectra of AK–HDH I depend on solvent conditions. In the absence of Gdn·HCl, the addition of the allosteric effector L-threonine causes a slight red shift of the maximum emission wavelength, from 332 to 336 nm, and a 20% decrease of the fluorescence intensity; these changes in fluorescence upon binding L-threonine have been previously used to follow the allosteric transition of AK–HDH I (Janin et al., 1969). The binding of L-threonine is known to stabilize the native tetrameric structure of AK–HDH I (Truffa-Bachi et al., 1968; Mackall & Neet, 1973; Cohen & Dautry-Varsat, 1980); therefore, the slight red shift does not result from an exposure to solvent of aromatic side chains but rather from a conformational change. The fluorescence emission spectrum of AK–HDH I is mainly due to its four tryptophan residues (Katinka et al., 1980) which are shielded from solvent in the absence of Gdn·HCl, whether L-threonine is bound or not. In 6 M Gdn·HCl, i.e., in strongly denaturing conditions, the fluorescence emission spectrum of AK–HDH I corresponds to that of tryptophan residues in contact with the aqueous solvent, a markedly lower intensity at 335 nm and a maximum emission wavelength around 350 nm (Teale & Weber, 1957); in 6 M Gdn·HCl, AK–HDH I has lost its structure and is completely unfolded. No measurement of fluorescence can be carried out in Gdn·HCl concentrations up to 2 M because of protein aggregation. At intermediate denaturant concentrations, 2–3 M Gdn·HCl, the fluorescence

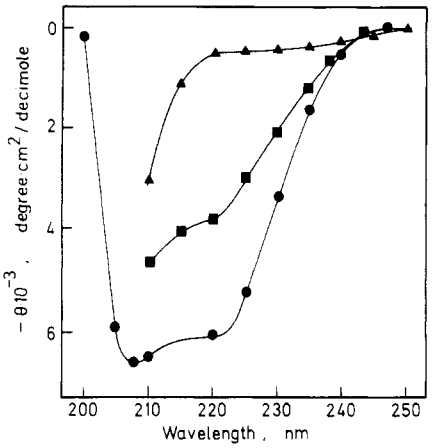

FIGURE 1: CD spectra of AK–HDH I under different solvent conditions. Protein concentration was 40 μg/mL (0.46 μM). The buffer is 0.1 M potassium phosphate, 0.5 M KCl, and 10 mM DTT at pH 7.2. (●) Native protein; (■) protein in the presence of 3 M Gdn·HCl; (▲) protein completely unfolded in 6 M Gdn·HCl.

emission spectrum of AK–HDH I is very close to that of the native enzyme in the presence of L-threonine. This suggests that, in 3 M Gdn·HCl, AK–HDH I retains a structure which is capable of shielding the tryptophan residues as efficiently (or almost) as in the native tetrameric conformation.

Figure 1 shows the CD spectra obtained for AK–HDH I under the same solvent conditions as those used for fluorescence measurements. In the absence of denaturant, the native protein has a spectrum which indicates significant amounts of secondary structure, α-helices and β-strands (Greenfield & Fasman, 1969; Chen et al., 1972). In 6 M Gdn·HCl, the secondary structure is completely destroyed, as judged from the CD spectrum, especially from the disappearance of the trough at 220 nm. At an intermediate concentration of 3 M Gdn·HCl, the CD spectrum is characteristic of a definite secondary structure; about 60% of the ellipticity at 220 nm is still present when the Gdn·HCl concentration is raised from 0 to 3 M (Figure 1). CD measurements therefore show that a stable structure, with an organized backbone conformation, exists in AK–HDH I in 3 M Gdn·HCl. This structure is also compact enough so as to bury its tryptophan residues in a hydrophobic environment. It can thus be concluded that at 3 M Gdn·HCl, where AK–HDH I is both inactive and soluble, the protein is in a conformation which has retained a significant part of its native structure.

Cooperative Unfolding–Refolding of the Residual Structure of AK–HDH I. Both fluorescence and CD measurements can be carried out on AK–HDH I in the presence of 2–6 M Gdn·HCl. When the Gdn·HCl concentration is raised from 2–3 M, where partly folded AK–HDH I exists, to 6 M, where the polypeptide chain is unfolded, a definite cooperative transition can be observed, either by fluorescence (Figure 2) or by CD (Figure 3). The same transition curve is obtained from the Gdn·HCl dependence of the maximum emission wavelength, the fluorescence intensity (Figure 2), or the ellipticity at 222 nm (Figure 3). In addition, complete reversibility is obtained for this transition.

The changes in any of these parameters occur abruptly, between 3 and 4 M, and show that extensive unfolding takes place in this Gdn·HCl concentration range. The midpoint of this transition is around 3.6 M Gdn·HCl, which shows that this residual structure has a remarkable stability toward Gdn·HCl-induced unfolding; it is indeed comparable to that of small disulfide-bonded monomeric proteins such as ribonuclease A and lysozyme (Tanford, 1968). The transition from

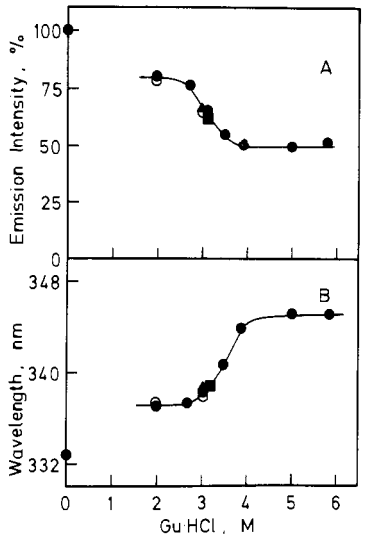

FIGURE 2: Unfolding–refolding transition curves of AK–HDH I measured by the changes in fluorescence intensity at λ_{EM} = 335 nm (A) and maximum emission wavelength (B). The excitation wavelength λ_{EXC} = 280 nm. Buffer conditions as given in Figure 1. Open symbols, final values of refolding after 10 min of unfolding in 6 M Gdn·HCl; closed symbols, final values obtained in the unfolding direction upon incubation of the native protein at the given Gdn·HCl concentration; circles, protein concentration 17.5 μg/mL (0.2 μM), no threoine added; squares, protein concentration 17.5 μg/mL (0.2 μM), incubation in the presence of 5 mM L-threonine; triangles, protein concentration 5 μg/mL (0.06 μM), incubation in the absence of L-threonine.

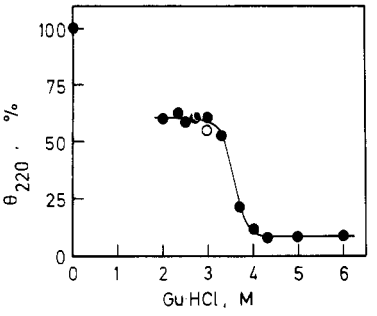

FIGURE 3: Unfolding–refolding transition curve of AK–HDH I measured by the changes in ellipticity at 220 nm. Protein concentration was 30 μg/mL (0.34 μM). All other specifications are as given in Figure 2.

native to completely unfolded AK–HDH I takes place in at least two steps; the first one occurs at a lower Gdn·HCl concentration, between 0.2 and 1.2 M, and is accompanied by a marked decrease in solubility, the loss of enzymatic activity (Müller & Garel, 1984), and the maintenance of a significant folded conformation. This step is sensitive to the presence of the allosteric ligand L-threonine. The second step takes place at a higher Gdn·HCl concentration, between 3 and 4 M, is independent of L-threonine (Figure 2), and involves the cooperative disruption of this residual structure.

Residual Structure Present in AK–HDH I in 3 M Gdn·HCl Is Probably That of a Folded Monomer. The preceding paper (Müller & Garel, 1984) suggests that the low Gdn·HCl transition is related to a dissociation of AK–HDH I into its constituting polypeptide chains. This conclusion is supported by several other results. First, the transition curve for the complete unfolding of the residual structure as obtained from fluorescence measurements (Figure 2) does not depend on AK–HDH I concentration. This transition occurs between the folded intermediate present in 2–3 M Gdn·HCl and the completely unfolded, and therefore monomeric, AK–HDH I chain; this transition should in principle depend on protein concentration, if it were related to an association–dissociation equilibrium, which is not observed.

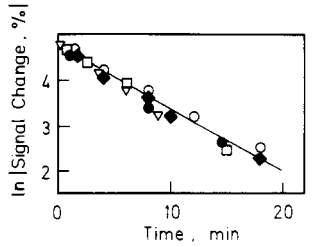

FIGURE 4: Semilogarithmic representation of refolding kinetics of AK–HDH I at 3 M final Gdn·HCl concentration. The buffer is 0.1 M potassium phosphate, 0.5 M KCl, and 10 mM DTT, at pH 7.2. The protein had been unfolded in the same buffer for 10 min at 6 M Gdn·HCl. Fluorescence intensity increase at 335 nm (excitation wavelength λ_{EXC} = 280 nm) in the absence of L-threonine at final protein concentrations of (▽) 5 μg/mL (0.06 μM), (●) 17.5 μg/mL, and (□) 50 μg/mL; (♦) fluorescence intensity increase in the presence of 5 mM L-threonine at a protein concentration of 17.5 μg/mL (0.2 μM); (○) ellipticity increase at 220 nm in the absence of L-threonine at 30 μg/mL (0.34 μM) protein. The slope of the straight line given corresponds to a monomolecular rate constant of 2.2×10^{-3} s^{-1} [half-life (τ) = 6.5 min].

The formation of this residual structure from unfolded and separated AK–HDH I chains can be kinetically followed by the changes in fluorescence intensity at 335 nm, upon a "jump" from 6 to 3 M Gdn·HCl; these changes obey first-order kinetics and give the same half-life independently of the protein concentration and of the presence of L-threonine (Figure 4). This in itself suggests that the kinetics of fluorescence changes are rate limited by a monomolecular reaction and therefore that the burial of tryptophan residues takes place within a single polypeptide chain; this step does not apparently involve the formation of an oligomeric structure. At 3 M final Gdn·HCl concentration, the half-life for forming this residual structure is about 7 min, and the same value is obtained from the changes in ellipticity at 220 nm (Figure 4); if the final Gdn·HCl concentration is decreased, the rate of folding is increased; at 2 M final Gdn·HCl concentration, the reaction becomes too fast to be measured by hand-mixing techniques, with a half-life certainly less than 0.3 min. This is true at any protein concentration used, down to 5×10^{-8} M in AK–HDH I chains; thus, if any bimolecular reaction governs the rate of fluorescence change, it should have a second-order rate constant greater than ca. 10^6 M^{-1} s^{-1}, which is close to the diffusion limit for molecules of the size of AK–HDH I chains (M_r ~90 000). This makes it quite likely that the structure, the formation of which shields the tryptophan residues from aqueous solvent, pertains to an isolated chain of AK–HDH I; the low Gdn·HCl transition would be a disruption of the quaternary structure which yields folded monomers, while the high Gdn·HCl unfolding would correspond to the destruction of the tertiary structure still present in these monomers. The influence of L-threonine on both transitions is also consistent with this interpretation. It is known that the allosteric ligand protects native tetrameric AK–HDH I against dissociation (Truffa-Bachi et al., 1968; Mackall & Neet, 1973; Cohen & Dautry-Varsat, 1980); this would explain the stabilization of the protein upon binding L-threonine toward the low Gdn·HCl transition (Müller & Garel, 1984). On the other hand, no monomeric species of AK–HDH I has ever been shown to bind the allosteric effector, in agreement with the threonine independence of the high Gdn·HCl transition (Figures 2 and 4).

Therefore, all the above arguments indicate that the interactions between subunits are much weaker than the interactions within a subunit toward the disruption by Gdn·HCl; the reversible cooperative transition given in Figures 2 and 3

would just measure the thermodynamic stability of the tertiary structure of an isolated AK–HDH I chain, without any contribution of quaternary structure.

Formation of this stable tertiary structure is fast at 2 M final Gdn·HCl concentration, and it should be even faster at a lower Gdn·HCl final concentration (Tanford, 1970). In the absence of denaturant, this folding reaction which occurs within a polypeptide chain will thus be faster than any of the reactions measured previously by the reappearance of enzymatic activity; the folded monomers, which are formed rapidly, will be inactive, and they will thus need a slower rearrangement reaction so that their kinase activity becomes efficient; indeed, the earliest step observed in reactivation is a first-order reappearance of the kinase activity (Garel & Dautry-Varsat, 1980a). These results therefore show that a folding step has to be added to the sequential mechanism proposed earlier for AK–HDH I (Garel & Dautry-Varsat, 1980b); this new folding step is the first in the self-assembly process of AK–HDH I from its unfolded chains.

A Fragment of AK–HDH I Corresponding to the Dehydrogenase Region (Two Chains of M_r 59 000 Each) Has Properties Very Close to Those of the Entire Protein. Limited proteolysis of AK–HDH I yields a fragment possessing full dehydrogenase activity and hence called the HDH fragment; this derivative is made up of two chains of M_r 59 000 each and corresponds to the C-terminal region of AK–HDH I (Vêron et al., 1972). This HDH fragment contains three tryptophan residues out of the four present in the entire chain (Katinka et al., 1980). The emission spectrum of the HDH fragment is different in the absence and in the presence of 2 and 6 M Gdn·HCl. In native conditions, the maximum emission wavelength is 333 nm, i.e., that of tryptophan residues buried in the protein; L-threonine has no influence on the emission spectrum, in agreement with its lack of influence on the activity of this fragment (Vêron, 1974). It can be concluded that the removal of the N-terminal third of the AK–HDH I chain and the dissociation from a tetrameric into a dimeric structure do not result in the significant exposure of tryptophan residues, since they appear shielded from the aqueous solvent in the HDH fragment, as in native AK–HDH I. In 6 M Gdn·HCl, the HDH fragment is completely unfolded; the maximum emission wavelength is shifted to 346 nm, and the fluorescence intensity decreases markedly. This decrease is identical with that obtained with AK–HDH I, which confirms that the extent of shielding the tryptophan residues upon going from 6 to 0 M Gdn·HCl is the same in the fragment and in the entire protein. At a Gdn·HCl concentration of 2 M, however, the HDH fragment is still able to bury a large fraction of its tryptophan residues away from the solvent, as judged from both maximum emission wavelength and intensity. It seems then as if this fragment retains some folded structure in 2 M Gdn·HCl.

The CD spectra of the HDH fragment resemble that of AK–HDH I; in native conditions (no Gdn·HCl), the spectrum shows the presence of a high level of secondary structure, which is totally absent in 6 M Gdn·HCl. In 2 M Gdn·HCl, the spectrum indicates the presence of a significant amount of residual structure, especially by the large ellipticity at 220 nm. As in the case of entire AK–HDH I, both fluorescence and CD measurements show that in 2 M Gdn·HCl, the HDH fragment possesses a folded structure which is capable of both burying its tryptophan residues and organizing its polypeptide backbone into a regular secondary structure (data not shown).

This folded structure of the HDH fragment is stable between 2 and 2.7 M Gdn·HCl; between 2.7 and 4 M Gdn·HCl,

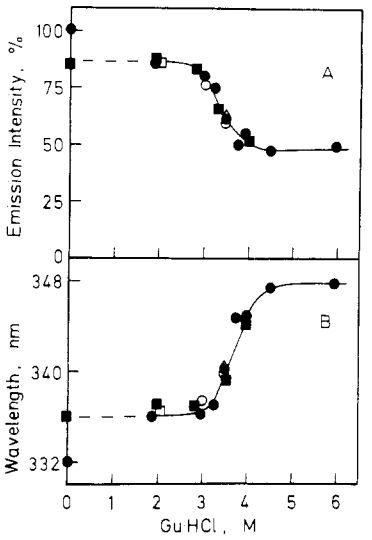

FIGURE 5: Unfolding–refolding transition curves of the dehydrogenase fragment as measured by the changes in fluorescence intensity at λ_{EM} = 335 nm (A) and maximum emission wavelength (B). Conditions are as given in Figure 1. Open symbols, final values of refolding after 10 min of unfolding at 6 M Gdn·HCl; filled symbols, final values obtained upon incubation of the native protein at the given concentration of denaturant; triangles, circles, and squares correspond to protein concentrations of 5 μg/mL (0.09 μM), 17.5 μg/mL (0.3 μM), and 50 μg/L (0.86 μM), respectively.

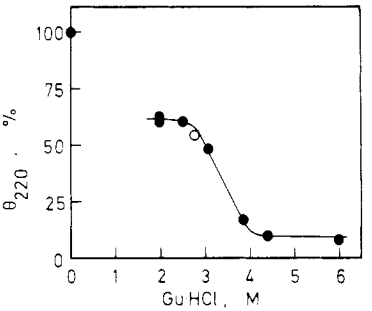

FIGURE 6: Unfolding–refolding transition curve of the dehydrogenase fragment as measured by the changes in ellipticity at 220 nm. Protein concentration was 30 μg/mL (0.52 μM). All other specifications are as given in Figure 5.

it unfolds according to a characteristic cooperative transition, whether monitored by its fluorescence (Figure 5) or CD (Figure 6) properties. This transition shows complete reversibility between 2 and 6 M Gdn·HCl; its midpoint corresponds to 3.4 M Gdn·HCl. The HDH fragment therefore possesses the same (or almost) remarkable stability toward Gdn·HCl-induced unfolding as the native protein; this shows that the N-terminal third of the chain does not significantly contribute to the stability of the tertiary structure, i.e., that the C-terminal region of the polypeptide chain folds and unfolds independently of the N-terminal part. It is probable that the interactions between subunits are not stronger in the HDH fragment than in native AK–HDH I and therefore that the folded state present between 2 and 3 M Gdn·HCl is monomeric in both cases. Indeed, the transition curve does not depend on the HDH fragment concentration (Figure 5). The rate of formation of this stable structure could not be studied by "jumping" the HDH fragment from 6 to 2.5 M Gdn·HCl, because the fluorescence change is too fast to measure. In the case of the HDH fragment also, a fast folding step exists and precedes the reactions observed earlier by activity measurements (Dautry-Varsat & Garel, 1981).

A Fragment of AK–HDH I Corresponding to the Kinase Region (M_r 27 000) Shows Cooperative Unfolding. The

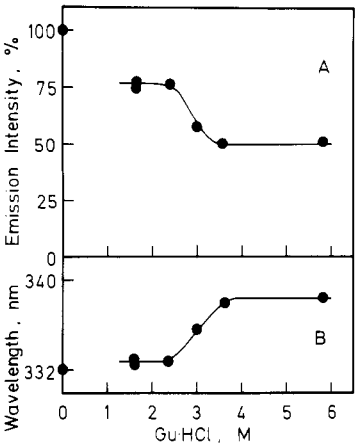

FIGURE 7: Unfolding of the kinase fragment by Gdn·HCl as measured by the changes in fluorescence intensity at 335 nm (A) and maximum emission wavelength (B). Protein concentration was 10 μg/mL (0.38 μM). Excitation wavelength = 280 nm. Buffer conditions are as given in Figure 1.

N-terminal third of the AK–HDH I chain can be isolated by limited proteolysis as a monomeric species possessing a weak kinase activity (Fazel et al., 1983). This AK fragment has only one tryptophan residue which is shielded from solvent in the absence of denaturant, as seen for a maximum emission wavelength of 332 nm. This wavelength increases to 338–339 nm when the Gdn·HCl concentration is raised from 2.5 to 3.5 M (Figure 7); at the same time, the fluorescence intensity at 335 nm decreases. No changes in either wavelength or intensity are observed between 3.5 and 6 M Gdn·HCl (Figure 7), which indicates that the AK fragment is completely unfolded in this concentration range of denaturant. However, the value of 338–339 nm for the maximum emission wavelength is slightly too short for a tryptophan residue in contact with the aqueous solvent (Teale & Weber, 1957); inspection of the amino acid sequence around the unique tryptophan of the AK fragment does not reveal any particular feature (Katinka et al., 1980). The unfolding transition of the AK fragment shows a definite cooperative behavior (Figure 7) and is largely reversible. The observed changes are smaller, but it seems, as in the preceding cases, that around 2 M Gdn·HCl this AK fragment exists as a folded species; this species loses its structure with a midpoint around 3 M Gdn·HCl. It is difficult to compare the stability of the structure formed by the AK fragment with that of the same segment of the intact AK–HDH I chain because of the unequal distribution of one tryptophan in the AK region and three in the complementary part (Katinka et al., 1980; Fazel et al., 1983). Indeed, an equimolar mixture of the two complementary fragments, AK of M_r 27 000 and HDH of M_r 59 000, would have an unfolding transition curve similar, within experimental errors, to that of an entire AK–HDH I chain of M_r 89 000. The interactions between the kinase and dehydrogenase regions in entire AK–HDH I should bring the same stabilization, if any, to either region; no such stabilization is found for the dehydrogenase region (see above), and thus the kinase region also is as stable when isolated in the AK fragment as when part of the intact chain.

Conclusions

The results presented here and in the preceding paper (Müller & Garel, 1984) show that the quaternary structure of AK–HDH I is destroyed by Gdn·HCl before the tertiary structure. At moderate Gdn·HCl concentrations, around 2–3 M, AK–HDH I exists in a monomeric folded state; a further increase in the denaturant concentration results in the cooperative unfolding of this folded state into a structureless chain. The major contribution to the total free enthalpy of stabilization of the native conformation of a given chain seems to be provided by the interactions within the chain itself and not by the interactions with the other chains. There is no strong energetic coupling between the tertiary and quaternary structures in AK–HDH I.

The folded compact structure which is stable in an isolated chain is formed rapidly when AK–HDH I is diluted out of the denaturant. The extrapolation to strongly native conditions suggests that this structure corresponds to an intermediate which appears during the self-assembly of AK–HDH I before any activity can be detected (Garel & Dautry-Varsat, 1980b). The formation of this intermediate is the major folding step of the overall process; a slow isomerization within this folded monomer results in the recovery of the kinase activity, and two subsequent association reactions yield the native enzyme with its catalytic and regulatory properties (Garel & Dautry-Varsat, 1980a). Therefore, not only is this compact structure important for the stabilization of the native conformation but also it appears as a crucial step in the formation of native AK–HDH I: there is apparently no strong kinetic coupling between the formation of the tertiary and quaternary structures.

The HDH fragment, lacking the N-terminal third of the polypeptide chain, behaves very similarly to the entire AK–HDH I. This shows that the missing part, i.e., the kinase region (Fazel et al., 1983), has no influence on the ability of the C-terminal segment, i.e., the dehydrogenase region, to fold up. Also, the AK fragment is able to exist in a compact folded conformation by itself; its stability toward unfolding by Gdn·HCl is not far from that of the complete chain. Therefore, in an isolated chain of AK–HDH I, the two compact regions corresponding to the N- and C-terminal moieties represent truly independent folding units. Moreover, the stability of the HDH fragment toward Gdn·HCl-induced unfolding is the same as that of the entire chain, as if no interaction occurs between these two folding units. There is apparently no structural coupling between the N- and C-terminal folding units in an isolated monomer of AK–HDHI; the kinase and dehydrogenase compact regions behave as if the only significant interaction between them was the covalent link represented by the flexible polypeptide segment which contains the cleavage site. It is possible that the destruction of the interactions between the subunits upon raising the Gdn·HCl concentration from 0 to 2 or 3 M is also accompanied by the destruction of the interactions between the compact regions within a subunit. Previous studies of the refolding process of AK–HDH I and of its fragments have suggested that the two compact regions were kinetically independent of each other: the rate at which either of them regains its native structure does not depend on its interactions with the other region (Garel & Dautry-Varsat, 1980; Dautry-Varsat & Garel, 1981). The present results indicate that these two regions are indeed thermodynamically independent folding units, since their unfolding transition curves were measured at equilibrium and are related to their intrinsic stability.

The overall native structure of AK–HDH I is the result of two stages of organization of the polypeptide chain: (a) the independent folding of (at least) two compact regions within a chain and (b) the formation of interactions between these folded regions, whether they belong to the same or different polypeptide chains. The first stage is responsible for the major energetic contribution to the folding process and

yields a solid tertiary frame, on which the second stage builds up the more subtle set of interactions involved in the biological activity. The same two-stage scheme probably applies to the folding of all large polypeptide chains. Indeed, results similar to those reported here for AK–HDH I have been observed with β-galactosidase (four chains of M_r 135 000 each) and the β_2 subunit of tryptophan synthase (two chains of M_r 55 000 each). The monomers of both β-galactosidase and β_2-tryptophan synthase consist of (at least) two compactly folded regions which can be isolated as fragments (Ullmann et al., 1968; Högberg-Raibaud & Goldberg, 1977a). In the case of β_2-tryptophan synthase, it was also shown that the isolated fragments are capable of refolding into a compact nativelike structure; interactions between those independent globular regions are needed for the expression of functional abilities (Högberg-Railbaud & Goldberg, 1977b). In the case of β-galactosidase, an enzymatically active protein is generated only upon the reassembly of the two complementary fragments (Goldberg, 1969). It is therefore likely that all large polypeptide chains fold up by parts and that such a folding mechanism is related to the evolution of these chains. AK–HDH I is thought to be the result of the fusion of two proteins (Katinka et al., 1980); the two independent folding regions could be the remains of these original proteins. Subsequent evolution would have added "minor" adjustments without grossly changing the original pattern in order to create more elaborate functional properties. AK–HDH I represents a good example of a self-assembly mechanism which still reflects the "molecular tinkering" performed by evolution (Jacob, 1981).

Acknowledgments

We are indebted to Dr. G. N. Cohen for constant interest and to Drs. M. E. Goldberg and M. Jullien for fruitful discussions. CD measurements were performed in the laboratory of Dr. R. Jaenicke at the University of Regensburg, FRG; Dr. R. Jaenicke's very kind hospitality and G. Zettlmeissl's help in making use of the spectropolarimeter are gratefully acknowledged. We also thank Y. Guillou and Dr. A. Fazel for collaboration in preparing AK–HDH I and its fragments.

Registry No. Gdn·HCl, 50-01-1.

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