Rogers, J., Hesketh, T. R., Smith, G. A., & Metcalfe, J. C. (1983) *J. Biol. Chem. 258*, 5994–5997.
Roos, A., & Boron, W. F. (1981) *Physiol. Rev. 61*, 296–433.
Rosoff, P. M., & Cantley, L. C. (1985) *J. Biol. Chem. 260*, 14053–14059.
Rothenberg, P., Glaser, L., Schlesinger, P., & Cassel, D. (1983) *J. Biol. Chem. 258*, 12644–12653.
Russel, J. M., & Boron, W. F. (1976) *Nature (London) 264*, 73–74.
Sandvig, K., Tønnessen, T. I., Sand, O., & Olsnes, S. (1986) *J. Biol. Chem. 261*, 11639–11644.
Schuldiner, S., & Rozengurt, E. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7778–7782.
Simchowitz, L., & Roos, A. (1985) *J. Gen. Physiol. 85*, 443–470.
Swann, K., & Whitaker, M. (1985) *Nature (London) 314*, 274–277.
Thomas, R. C. (1977) *J. Physiol. (London) 273*, 317–338.
Vigne, P., Frelin, C., & Lazdunski, M. (1984) *EMBO J. 3*, 1865–1870.
Vigne, P., Frelin, C., & Lazdunski, M. (1985) *J. Biol. Chem. 260*, 8008–8013.

Mechanism of Renaturation of a Large Protein, Aspartokinase–Homoserine Dehydrogenase†

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Received October 9, 1986; Revised Manuscript Received December 30, 1986

ABSTRACT: The renaturation of aspartokinase–homoserine dehydrogenase and of some of its smaller fragments has been investigated after complete unfolding by 6 M guanidine hydrochloride. Fluorescence measurements show that a major folding reaction occurs rapidly (in less than a few seconds) after the protein has been transferred to native conditions and results in the shielding of the tryptophan residues from the aqueous solvent; this step also takes place in the fragments and probably corresponds to the independent folding of different segments along the polypeptide chain. The reappearance of the kinase activity, which is an index of the formation of "native" structure within a single chain, is much slower (a few minutes) and has the following properties: (i) it is involved in a kinetic competition with the formation of aggregates; (ii) it has an activation energy of 22 ± 5 kcal/mol; (iii) it is not related to a slow reaction in unfolding and thus probably not controlled by the cis–trans isomerization of X–Pro peptide bonds; (iv) its rate is inversely proportional to the solvent viscosity. It seems as if this reaction is limited by the mutual arrangement of the regions that have folded rapidly and independently. It is proposed that the mechanism where a fast folding of domains is followed by a slow pairing of folded domains could be generalized to other long chains composed of several domains; such a slow pairing of folded domains would correspond to a rate-limiting process specific to the renaturation of large proteins. The reappearance of the dehydrogenase activity measures the formation of a dimeric species. The dimerization can occur only after each chain has reached its "native" conformation. This reaction has an activation energy of 6 ± 3 kcal/mol and is not influenced by the solvent viscosity; in this case, the reaction seems related to a minor conformational change occurring after dimerization.

The renaturation of an oligomeric protein from its unfolded and separated chains implies a mixture of folding and association reactions (Jaenicke, 1982, 1984; Jaenicke & Rudolph, 1980). In the case of aspartokinase–homoserine dehydrogenase (AK–HDH),[1] a tetrameric and bifunctional enzyme, the renaturation process could be decomposed into a succession of several steps (Garel & Dautry-Varsat, 1980a,b; Dautry-Varsat & Garel, 1981; Müller & Garel, 1984a): (i) a polypeptide chain acquires some organized structure, as seen from its fluorescence and circular dichroism, and forms a stable (partially) folded monomeric species; (ii) this (partially) folded species further isomerizes to yield an intermediate with full kinase activity and still a monomeric structure; (iii) two folded monomers then associate into a dimeric species that possesses a normal dehydrogenase activity; (iv) finally, two dimers associate to regenerate the native tetramer with all its catalytic and regulatory properties.

In the native state the polypeptide chain of AK–HDH is folded into three compact regions (Fazel et al., 1983). Two smaller fragments can be obtained from AK–HDH that correspond each to only two of these compact regions. The AK fragment is obtained from a nonsense mutant: it lacks the C-terminal region, is stable as a monomer, and has an intact kinase activity. The HDH fragment is obtained by limited proteolysis: it lacks the N-terminal region, has a dimeric structure, and possesses an intact dehydrogenase activity (Cohen & Dautry-Varsat, 1980). Both fragments can resume their functional structure after complete unfolding.

† This work was supported by the Centre National de la Recherche (UA 1129), the Institut Pasteur, and the Université Paris 6 (UER 58).
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[1] Abbreviations: AK–HDH, aspartokinase–homoserine dehydrogenase (EC 2.7.2.4 and EC 1.1.1.3); AK fragment, derivative of AK–HDH obtained by an ochre mutation of the corresponding gene; HDH fragment, derivative of AK–HDH obtained by limited proteolysis; Gdn·HCl, guanidine hydrochloride; EDTA, (ethylenedinitrilo)tetraacetic acid.

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The comparison between the kinetics of reactivation of the fragments and that of the entire chain shows that neither the mechanism nor the rate of renaturation is markedly affected by the removal of 30–40% at either end of the chain (Dautry-Varsat & Garel, 1981; Müller & Garel, 1984a). It seems then that, during the renaturation of AK–HDH, several segments along the polypeptide chain behave as independent folding units.

In order to analyze further the individual processes involved in the renaturation of AK–HDH, we have studied the temperature and viscosity dependences of two of the steps, those controlling the reappearance of the kinase and the dehydrogenase activities for both the entire protein and its two monofunctional AK and HDH fragments. Indeed, the value of the activation energy (as derived from the temperature dependence of the rate constant of a folding reaction) has already been used as an identification index, e.g., in the case of the cis–trans isomerization of the X–Pro peptide bonds (Brandts et al., 1975; Cook et al., 1979; Stellwagen, 1979; Garel, 1980). AK–HDH has 29 prolines (Katinka et al., 1980), and its refolding into an active monomeric species could be limited by the large number of possible isomers (Creighton, 1978).

The observation that the rate of a reaction depends on the viscosity of the solvent suggests that it is hydrodynamically controlled. A bimolecular reaction always involves the encounter between the two reagents, which is a diffusive process, but the overall rate may not be limited by that of free diffusion. A monomolecular reaction can also involve the relative diffusion of different parts of the same molecule and show some viscosity dependence (Shimada & Szwarc, 1975); for instance, the viscosity dependence of the rate of DNA renaturation suggests that the winding of DNA upon double-helix formation could be limited by the friction of coiled strands on solvent (Wetmur & Davidson, 1968). In the case of the reactivation of AK–HDH, some viscosity dependence is expected for all the steps that are rate-limited by significant relative displacements through the solvent of compact regions that have folded independently.

Materials and Methods

Most experimental methods have been described previously: the preparation of AK–HDH and of its fragments, the assays for the kinase and dehydrogenase activities, the denaturation and renaturation procedures, the composition of the various buffers, the kinetic analysis, etc. (Garel & Dautry-Varsat, 1980a; Dautry-Varsat & Garel, 1981; Müller & Garel, 1984a,b). The viscosity of the solvent was changed by adding either glycerol or sucrose, and the increase in viscosity was taken from the tabulated values given in the *Handbook of Chemistry and Physics* (1973).

The kinetics of reaction of the kinase activity were interpreted according to the unimolecular mechanism

$$U \xrightarrow{k} M$$

and the uni-bi mechanism was used for the reactivation of the dehydrogenase activity:

$$2U \xrightarrow{k_1} 2M \xrightarrow{k_2} D$$

U, M, and D are respectively the unfolded chain, the folded monomer with the kinase activity, and the dimer with the dehydrogenase activity. The rate constant k was directly determined from the monoexponential regain of kinase activity for AK–HDH (such as that shown in Figure 2) and its AK fragment; the rate constants k_1 and k_2 were obtained from the biphasic regain of dehydrogenase activity for AK–HDH (such as that shown in Figure 6) and its HDH fragment at different protein concentrations by computer fitting to the analytical solution (Chien, 1948).

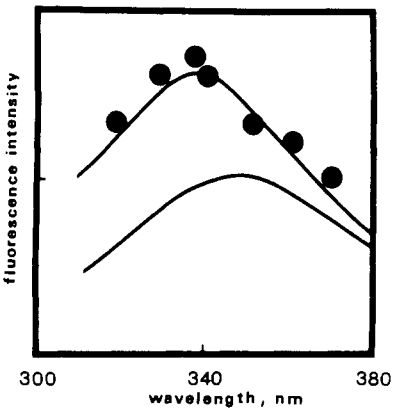


FIGURE 1: Amplitude of the rapid change in fluorescence intensity observed upon refolding of AK–HDH. The protein was diluted at least 100-fold from 6 M Gdn·HCl into the renaturation buffer, 0.1 M potassium phosphate, 0.5 M KCl, 0.01 M K_2MgEDTA, 0.01 M dithiothreitol, and 50 μg/mL bovine serum albumin, pH 7.2 (Garel & Dautry-Varsat, 1980a). The two emission spectra are those of the unfolded (lower) and folded (upper) AK–HDH chains (Garel et al., 1984). At each wavelength, the points correspond to the sum of the fluorescence intensity of the unfolded form and of the amplitude of the "burst" observed upon dilution. The actual burst in fluorescence was determined by extrapolation to zero time because some aggregation occurs at the high protein concentration of 25 nM; the increase in signal due to stray light is however slow enough to permit measurements.

Results and Discussion

The AK–HDH Monomer Folds in Two Steps: A Faster Reaction Corresponding to the Major Folding Process Is Followed by a Slower Isomerization That Restores the Kinase Activity. In 3 M Gdn·HCl, AK–HDH exists as a stable (partially) folded monomeric species as seen from its fluorescence and circular dichroism properties (Garel et al., 1984; Müller & Garel, 1984a). The formation of this folded monomer from the unfolded chain has a half-life of 6.5 min in 3 M Gdn·HCl and of less than 0.5 min in 2 M Gdn·HCl (Müller & Garel, 1984a). Upon dilution of AK–HDH from 6 M to less than 0.1 M Gdn·HCl, there is a fluorescence change too rapid to be measured after manual mixing; this holds even at lower temperature (down to 5 °C) or in the presence of glycerol [up to 30% (v/v)]. The amplitude of this rapid fluorescence change is consistent with the difference spectrum between the folded and unfolded monomers (Figure 1). There is also a large change in circular dichroism at 220 nm that takes place upon dilution of AK–HDH from 6 M to less than 0.1 M Gdn·HCl; its rate is too fast to measure, and its amplitude is about 60–80% of the difference between native and unfolded AK–HDH. Therefore, in less than 0.1 M Gdn·HCl, the process that leads to the shielding of tryptophan residues from aqueous solvent and to the buildup of the secondary structure occurs rapidly (at most within seconds). A similar fast reaction corresponding to the extensive formation of organized structure also takes place in both the AK and HDH fragments, as judged from similar rapid changes in fluorescence and circular dichroism signals.

Although this first rapid step represents a major part of the folding process, it yields inactive species. The reappearance of the kinase activity, which measures the formation of a functional monomer in AK–HDH or its AK fragment, takes place more slowly: at a temperature of 21 °C, the reactivation

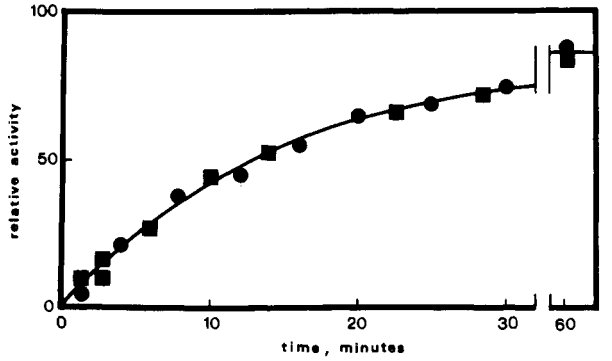


FIGURE 2: Kinetics of renaturation of AK–HDH measured by the regain of the kinase activity. The activity is expressed relative to that of the native enzyme at the same concentration of 2.4 nM in chains. AK–HDH was denatured by 6 M Gdn·HCl for at least 1 h at 50 °C (Garel & Dautry-Varsat, 1984a) (●) or for 30 s at 0 °C (■); the curve corresponds to a first-order reaction with a rate constant k of 10^{-3} s^{-1}. The temperature was 21 °C.

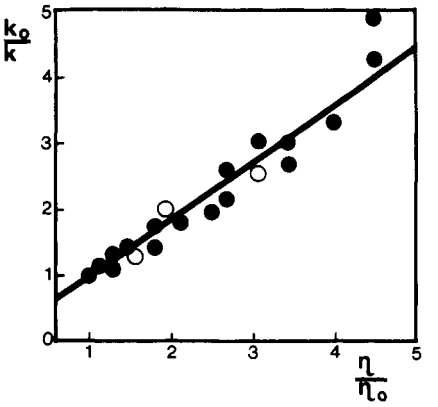


FIGURE 3: Dependence on solvent viscosity of the rate of reactivation of the kinase activity of AK–HDH. The bulk viscosity of the solvent is varied by increasing the fraction of either glycerol (●) or sucrose (○), leaving all the other components (except water) unchanged. The temperature is 21 °C, at which the rate constant for folding an AK–HDH chain in the absence of glycerol is $k_0 = 10^{-3}$ s^{-1} (Figures 2 and 3). The values of η/η_0 are taken from the *Handbook of Chemistry and Physics* (1973). The straight line has a slope of 0.92, i.e., corresponds to an almost inversely proportional relationship between k and η.

reaction has a half-life of 11.5 min for AK–HDH (Figure 2) and of 8.5 min for the AK fragment. The reactivation step is not correlated with a large change in either fluorescence or circular dichroism. It seems to correspond to an isomerization step that is of limited amplitude in terms of conformation and of crucial importance in terms of enzymatic activity. This isomerization occurs in an already folded state; it belongs to the overall folding process of AK–HDH not only because it controls the regain of kinase activity but also because it controls the reappearance of the ability to form dimeric species (see below).

The Slow Reactivation Step Is Strongly Dependent on the Temperature and on the Viscosity of the Solvent. The rate at which the kinase activity reappears upon refolding of AK–HDH or its AK fragment depends markedly on temperature with an activation energy of 22 ± 5 kcal/mol for the intact chain and of 18 ± 5 kcal/mol for the fragment.

The rate at which the kinase activity reappears upon refolding of AK–HDH depends quite strongly on the viscosity of the solvent; Figure 3 shows that there is an almost inversely proportional relationship between the relative changes in the viscosity and those in the first-order rate constant of reactivation. The viscosity of the solvent was changed by adding either glycerol [up to 42% (v/v)] or sucrose (up to 1 M), and the change in the rate of reactivation depends on the final viscosity and not on the nature of the additive (Figure 3). The presence of glycerol (or sucrose to a lesser extent) not only affects the bulk viscosity of the solvent, but it also stabilizes the structure of proteins (Gekko & Timasheff, 1981; Arakawa & Timasheff, 1982). It seems however that the renaturation of AK–HDH is not markedly influenced by the stabilizing effect of glycerol (or sucrose) for the following reasons:

(i) The presence of another stabilizing agent, the sulfate ion (up to 0.5 M), does not result in any significant increase in the rate of reactivation. Yet sulfate is generally more efficient on a molar basis than glycerol as a stabilizing agent: for instance, 1 M sulfate raises the melting temperature of ribonuclease at neutral pH by 7–8 deg (von Hippel & Wong, 1965) as compared to a 1–2-deg raise with 1 M glycerol (Gerlsma & Stuur, 1972). A control experiment showed that there is little if any change in the rate of reactivation upon raising the ionic strength and that there is no difference whether sulfate or chloride is used as an added anion; the lack of sulfate effect is not due to a compensation between an ionic and a stabilizing contribution.

(ii) Would glycerol favor the folded state, it should make the reactivation of AK–HDH faster, which it does not (Figure 3); the possibility that glycerol decreases the rate of reactivation by stabilizing selectively incorrectly folded and inactive intermediates cannot be excluded but will not be considered here because of the lack of experimental evidence.

(iii) It is quite unlikely that the lack of glycerol dependence of the rate of reappearance of the dehydrogenase activity (Figure 5) results from an exact compensation between the opposite effects of stabilization and viscosity.

The conclusion is that the rate of the slow reactivation of AK–HDH is much more influenced by the viscosity of the solvent rather than by its stabilizing properties, and thus that this reaction which occurs within a single chain is hydrodynamically controlled. The reactivation of the AK fragment also depends on the viscosity of the solvent as seen from the decrease of its rate in the presence of glycerol; the C-terminal segment is apparently not involved in the process leading to the kinase reactivation.

The Slow Reactivation Step Is Probably Not Rate Limited by the Cis–Trans Isomerization of X–Pro Peptide Bonds. A value around 20 kcal/mol for the activation energy of the slow refolding reaction of a monomeric protein has sometimes been associated with the cis–trans isomerization of X–Pro peptide bonds (Brandts et al., 1975; Cook et al., 1979; Stellwagen, 1979; Garel, 1980). The rate of isomerization between the cis and trans configurations of proline residues in unfolded ribonuclease is independent of the concentration of Gdn·HCl between 2 and 6 M (Schmid & Baldwin, 1979) and thus also of solvent viscosity, which varies in this range. Therefore, the reactivation of AK–HDH or its AK fragment differs from the cis–trans isomerization of X–Pro peptide bonds in unfolded ribonuclease: the former depends on solvent viscosity, and the latter does not.

The slow refolding reactions that are limited by the cis–trans isomerization of proline residues are always related to a slow reaction in unfolding that corresponds to the relaxation of each X–Pro peptide bond from the defined cis or trans configuration that it has in the native state to an equilibrium mixture in the unfolded state (Brandts et al., 1975). The presence of such a slow unfolding reaction can be tested by "double-jump" experiments: the protein is first unfolded in strongly denaturing conditions for a given time and then refolded to completion; the fraction of "slow-refolding" material is measured as a function of the time allowed for unfolding. The same

kinetics of reactivation are obtained independently of the duration of unfolding by 6 M Gdn·HCl, between 30 s and 1 h, even when unfolding is carried out at 0 °C [where the relaxation of proline residues in an unfolded chain takes several minutes (Brandts et al., 1975; Nall et al., 1978; Schmid & Baldwin, 1979; Garel, 1980)] (Figure 2). In all the conditions studied, the totality of the protein was under a "slow-reactivating" form; no evidence for the presence of active enzyme at the beginning of refolding was obtained. Therefore, formation of the slow-refolding species of AK–HDH is rapid and takes less than 30 s at 0 °C; the half-life of this reaction is even shorter than about 12 s, assuming that we would have detected unambiguously an initial level of activity of 20% of the final value. It seems then that the slow reactivation of AK–HDH is not related to a slow unfolding reaction and therefore not governed by the cis–trans isomerization of X–Pro peptide bonds.

The Slow Reactivation Step Probably Corresponds to the Pairing of Already Folded Regions. The slow reactivation step takes place within an already folded state; it is not limited by the cis–trans isomerization of proline residues, its rate is controlled by the viscous friction of the solvent, and it governs the reappearance of the kinase activity. The following arguments are in agreement or even support the conclusion that this slow folding process of a single chain could be the pairing or correct adjustment of already folded regions.

(i) Many large polypeptide chains appear as composed of several compact regions, which are supposed to correspond to independent folding segments (Wetlaufer, 1973; Schulz & Schirmer, 1979; Janin & Wodak, 1983); this is indeed the case with AK–HDH in which three compact regions exist (Fazel et al., 1983) and for which the presence of independently folding units has been shown (Dautry-Varsat & Garel, 1981; Müller & Garel, 1984a).

(ii) The folding of a segment of 100–150 amino acids could be very rapid; indeed, a small protein can find the way to its completely native state in less than a tenth of a second, as was first shown for ribonuclease (Garel & Baldwin, 1973; Garel et al., 1976).

(iii) It is thus likely that the first rapid step in which the tryptophan residues become buried and the secondary structure is formed corresponds to the independent folding of various segments along the polypeptide chain.

(iv) This folding of independent regions is however not sufficient to generate the kinase activity. When isolated, the N-terminal region of AK–HDH can reversibly unfold and refold (Müller & Garel, 1984a), but it has less than 0.5% kinase activity (Fazel et al., 1983; Vêron et al., 1985); the middle region is completely inactive (Fazel et al., 1983). In both AK–HDH and its AK fragment, the activity seems then to depend on a proper adjustment of the two N-terminal and middle regions; this adjustment or pairing would be slow and limit the rate of reactivation independently of the presence or absence of the C-terminal 40% of the chain.

(v) The viscosity dependence of the rate of reactivation suggests that the slow pairing of already folded regions is limited by their friction on the solvent; this pairing step would then involve some relative movement of these regions. The reciprocal relationship between the rate of pairing and the viscosity of solvent is indeed that expected from the simple Einstein–Stokes model of diffusive movements (translational and/or rotational) of large rigid bodies through an isotropic continuous solvent.

(vi) It is known that in native AK–HDH the N-terminal and middle regions are separated by a freely accessible segment

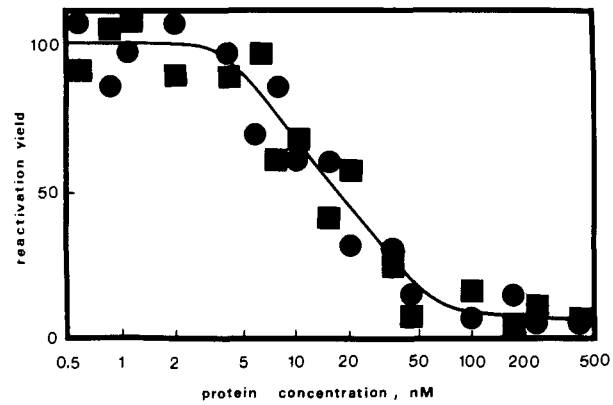
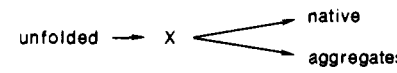


FIGURE 4: Dependence of the extent of reactivation measured for kinase (●) and dehydrogenase (■) activities on concentration of protein. AK–HDH concentration is expressed as chain concentration. The extent of reactivation was determined after 1 h for the kinase and by extrapolation to infinite time (Garel & Dautry-Varsat, 1980a) for the dehydrogenase.

of 7–10 residues (Sibilli et al., 1981). The same slow pairing reaction occurs in AK–HDH and its AK fragment and could thus be due to the relative movements of these two compact regions connected by a short flexible hinge (residues 292–301).

The Slowness of the Correct Intrachain Pairing Reaction between Folded Domains Results in a Kinetic Competition with Incorrect Interchain Interactions That Lead to Aggregation. The yield in the reactivation of the kinase activity measures the fraction of the protein chains that are able to resume their functional structure after complete unfolding. As in the case of many proteins (Jaenicke, 1984), this extent of reactivation depends markedly on the concentration of AK–HDH chains at which renaturation occurs: up to about 5 nM all the activity can be regained, between 5 and 50 nM this extent decreases, and above 50 nM almost no activity can be recovered (Figure 4). This is usually interpreted as resulting from a kinetic competition between folding and aggregation at some branch point X (Jaenicke, 1982, 1984; Garel et al., 1984; Müller & Garel, 1984b):



The same concentration dependence of the extent of reactivation is obtained for the kinase activity, which corresponds to the folding of a monomer, and for the dehydrogenase activity, which is related to a dimeric species (Figure 4). Thus, the branch point X is located before or at the pairing step that controls the kinase reactivation, and all the protein that has reached the correct monomer structure will proceed further to the native tetramer. Aggregation therefore does not compete with the normal association steps of the renaturation pathway of AK–HDH but only with the reactions that occur within a single chain. After they have folded independently and rapidly, the different domains of the chain must interact to complete the tertiary structure of the monomer. However, the same folded regions exist on each chain, and a given domain cannot distinguish between a partner belonging to the same or to a different polypeptide chain (Goldberg & Zetina, 1980). The slower the intramolecular pairing reaction, the longer the lifetime of folded and unpaired domains and the more probable the formation of stable intermolecular interactions that produce aggregates. The two-step mechanism fast domain folding/slow domain pairing is responsible for the kinetic competition that reduces the renaturation yield even at the rather low protein concentration of 10 nM or so.

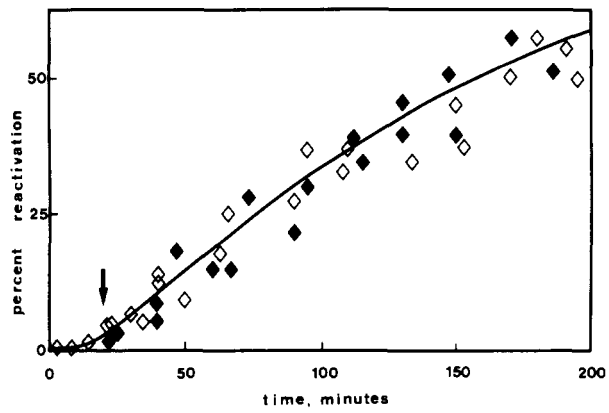


FIGURE 5: Time course of regain of dehydrogenase activity upon renaturing AK–HDH in the absence (◇) or in the presence (◆) of 25% (v/v) glycerol, at a temperature of 21 °C and at a concentration of 3.5 nM in AK–HDH chains. The renaturation of AK–HDH is carried out in two steps: first, AK–HDH is allowed to renature for 20 min in a glycerol-free buffer; then, it is split into two samples (arrow), one with and one without glycerol, for further reactivation. This procedure gets rid of most of the influence of glycerol on the lag phase, and the activities of the two samples at various times can be directly compared. The curve corresponds to a uni-bi mechanism with the rate constant $k_1 = 10^{-3}$ s^{-1} and $k_2 = 3.8 \times 10^4$ M^{-1}·s^{-1} (note that the rate constant k_1 has the same value as that given in Figure 2 from kinase activity measurements). Computer simulations show that a value of 3×10^4 M^{-1}·s^{-1} is the lowest value for k_2 that is still (hardly) consistent with the experimental data: 25% (v/v) glycerol, which corresponds to a factor 2.2 in viscosity, does not affect k_2 by more than 1.25, if at all.

The Reappearance of the Dehydrogenase Activity Is Probably Due to an Isomerization Step of Small Amplitude after the Formation of Dimeric Species. The reappearance of the dehydrogenase activity upon reactivation of AK–HDH or its HDH fragment follows biphasic kinetics (Figure 5). It can be described by the uni-bi mechanism given above.

The value of the first-order rate constant k_1 calculated by curve fitting of kinetics such as that shown in Figure 5 is similar to that of the first-order rate constant k measured from the kinase reactivation (Figure 2). This holds for different values of temperature and solvent viscosity, showing that the same reaction controls the reappearance of the kinase activity and of the ability to dimerize. This reaction takes place in AK–HDH and its AK and HDH fragments and thus implies the middle region which they have in common.

The second-order rate constant k_2 of the dimerization step shows only a moderate dependence on temperature, corresponding to an activation energy of 6 ± 3 kcal/mol. The value of about 5×10^4 M^{-1}·s^{-1} obtained around 25 °C is much smaller than that estimated for a diffusion-controlled reaction, which shows that the rate of encounter between two chains is not limiting for the formation of active dimers (Hammes, 1978).

The presence of glycerol [up to 25% (v/v)] modifies the lag phase because an increase in solvent viscosity lowers the value of k_1. The two-step renaturation procedure described in the legend of Figure 5 allows minimization of the influence of glycerol on the first monomolecular reaction, from U to M. It can be seen in Figure 5 that the presence of 25% (v/v) glycerol does not change the bimolecular part of the reactivation kinetics of the dehydrogenase, thus indicating that the rate constant k_2 of the dimerization reaction, from 2M to D, does not significantly depend on the viscosity of the solvent. That a bimolecular reaction between two species A and B obeys second-order kinetics and is independent of the solvent viscosity can be explained as follows. First A and B diffuse through the solvent and collide to form an encounter complex A*B; this encounter complex A*B is highly unstable and can either dissociate into separate A and B or isomerize into a stable complex A·B:

$$A + B \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} A^*B \xrightarrow{k_{iso}} A{\cdot}B$$

The rates of the formation of A*B, k_{on}, and its dissociation into A and B, k_{off}, depend similarly on the solvent viscosity because they are both controlled by the diffusion of A and B, toward or apart from each other; the ratio k_{on}/k_{off} is thus independent of viscosity. When the rate of formation of A·B from A*B is much smaller than that of dissociation of A*B (i.e., when $k_{iso} \ll k_{off}$) and when the concentration of A*B is constant with time (i.e., when a steady state is established), the apparent rate for forming A·B from A and B is given by

$$d[A{\cdot}B]/dt = k_{iso}(k_{on}/k_{off})[A][B]$$

The appearance of A·B follows second-order kinetics, but the corresponding rate constant will depend on solvent viscosity only if k_{iso} does; there is no longer any viscosity dependence due to the encounter reaction because the rate of formation of A·B is governed by the amount of A*B and not by the rate of its formation.

The regain of dehydrogenase activity follows second-order kinetics, thus showing that it occurs together with or after the formation of dimeric species. It is apparently not limited by the rate of relative displacements of the two folded monomers through the solvent; indeed, the second-order constant k_2 is much smaller than that for a diffusion-limited encounter. It does not seem either to be limited by a conformational change occurring after the encounter step and having a large enough amplitude to be sensitive to the solvent viscosity. Therefore, it is likely that the reactivation of the dehydrogenase is related to minor structural rearrangements within a dimer and not to major relative displacements of the folded regions; these rearrangements could be similar to the subtle conformation changes of the protein that are apparently needed for a normal dehydrogenase activity in native AK–HDH (Müller & Garel, 1984c).

Conclusions

The succession of events that can be proposed from this and previous work (Garel & Dautry-Varsat, 1980a,b; Dautry-Varsat & Garel, 1981; Müller & Garel, 1984a) for the process producing native AK–HDH from its unfolded and separated chains can be compared to the results obtained with other domain-containing proteins:

(i) Independent segments of the polypeptide chain fold rapidly into compact regions in which the backbone is organized into elements of secondary structure and which are stable enough to expel water and to shield the aromatic groups from aqueous solvent. A similar fast reaction has also been observed by fluorescence and/or circular dichroism as the first folding step in several cases (Jaenicke & Rudolph, 1980; Crisanti & Matthews, 1981; Zetina & Goldberg, 1982; Zettlmeissl et al., 1984).

(ii) No reaction associated with the cis–trans isomerization of X–Pro peptide bonds has been yet observed with AK–HDH, whereas such a reaction has been detected with the α-chain of tryptophan synthase (Crisanti & Matthews, 1981; Beasty et al., 1986) and with octopine dehydrogenase (Teschner et al., 1987): this reaction is faster than the complete folding of the chain. It seems also that the slow folding reaction of penicillinase is not limited by the cis–trans isomerization of proline residues (Creighton & Pain, 1980).

(iii) The folded regions diffuse relative to each other and slowly pair to yield the "native" state of a chain. That it is

this domain pairing reaction which is rate limiting for the proper folding of a chain has also been proposed for the α-chain of tryptophan synthase (Beasty et al., 1986) and for octopine dehydrogenase (Teschner et al., 1987). It could be that the slow pairing of folded domains is a general rate-limiting step in the folding of large chains.

(iv) Two "native" monomers encounter and stick together to form a dimeric species. Such a step must exist in the self-assembly of all oligomeric proteins.

(v) A minor conformational change within the dimer generates the dehydrogenase activity. The β_2-subunit of tryptophan synthase also acquires it functional state upon a small conformational change after dimerization (Blond & Goldberg, 1985).

(vi) Another conformational change results in the sensitivity of the dehydrogenase active sites to the allosteric inhibition by threonine.

(vii) Two functional dimers associate into a tetrameric species, which then acquires the allosteric regulation by threonine of its kinase active sites.

Some of these steps are observed in several proteins, which suggests that a similar sequence of reactions is a general mechanism for the renaturation of large and complex proteins. The various intermediates involved in this mechanism seem to be related to conformational processes that have smaller and smaller amplitudes as renaturation proceeds, with large changes at the beginning and minor readjustments toward the end. The subtle structural rearrangements are nevertheless accompanied by important effects on the functional properties.

ACKNOWLEDGMENTS

J.-R.G. is indebted to Drs. R. Jaenicke, R. E. K. Rudolph, and F. X. Schmid and their colleagues of the University of Regensburg (FRG) for their hospitality and their cooperation during the various phases of this work and to Dr. R. L. Baldwin for his constructive comments. We gratefully acknowledge the support of Alexander von Humboldt Stiftung, which made the collaboration with the Regensburg group possible.

REFERENCES

Arakawa, T., & Timasheff, S. N. (1982) *Biochemistry 21*, 6536–6544.

Beasty, A. M., Hurlé, M. R., Mauz, J. T., Stackhouse, T., Onuffer, J. J., & Matthews, C. R. (1986) *Biochemistry 25*, 2965–2974.

Blond, S., & Goldberg, M. E. (1985) *J. Mol. Biol. 182*, 597–606.

Brandts, J. F., Halvorson, H. R., & Brennan, M. (1975) *Biochemistry 14*, 4953–4963.

Chien, J. Y. (1948) *J. Am. Chem. Soc. 70*, 2256–2261.

Cohen, G. N., & Dautry-Varsat, A. (1980) in *Multifunctional Proteins* (Bisswanger, H., & Schmincke-Ott, E., Eds.) pp 49–121, Wiley, New York.

Cook, K. H., Schmid, F. X., & Baldwin, R. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6157–6161.

Creighton, T. E. (1978) *J. Mol. Biol. 125*, 401–406.

Creighton, T. E., & Pain, R. H. (1980) *J. Mol. Biol. 137*, 431–436.

Crisanti, M. M., & Matthews, C. R. (1981) *Biochemistry 20*, 2700–2706.

Dautry-Varsat, A., & Garel, J. R. (1981) *Biochemistry 20*, 1396–1401.

Fazel, A., Müller, K., Le Bras, G., Garel, J. R., Vêron, M., & Cohen, G. N. (1983) *Biochemistry 22*, 158–165.

Garel, J. R. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 795–798.

Garel, J. R., & Baldwin, R. L. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3347–3351.

Garel, J. R., & Dautry-Varsat, A. (1980a) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3379–3383.

Garel, J. R., & Dautry-Varsat, A. (1980b) in *Protein Folding* (Jaenicke, R., Ed.) pp 485–499, Elsevier, Amsterdam.

Garel, J. R., Nall, B. T., & Baldwin, R. L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1853–1857.

Garel, J. R., Martel, A., Müller, K., Ikai, A., Morishima, N., & Sutoh, K. (1984) *Adv. Biophys. 18*, 91–113.

Gekko, K., & Timasheff, S. N. (1981) *Biochemistry 20*, 4667–4676.

Gerlsma, S. Y., & Stuur, E. R. (1972) *Int. J. Pept. Protein Res. 4*, 377–383.

Goldberg, M. E., & Zetina, C. R. (1980) in *Protein Folding* (Jaenicke, R., Ed.) pp 469–484, Elsevier, Amsterdam.

Hammes, G. G. (1978) *Principles of Chemical Kinetics*, pp 65–67, Academic, New York.

Handbook of Chemistry and Physics (1973) 54th ed., pp D203 and D229, CRC Press, Cleveland, OH.

Jaenicke, R. (1982) *Biophys. Struct. Mech. 8*, 231–256.

Jaenicke, R. (1984) *Angew. Chem., Int. Ed. Engl. 23*, 395–413.

Jaenicke, R., & Rudolph, R. (1980) in *Protein Folding* (Jaenicke, R., Ed.) pp 525–548, Elsevier, Amsterdam.

Janin, J., & Wodak, S. (1983) *Prog. Biophys. Biol. Mol. 42*, 21–78.

Katinka, M., Cossart, P., Sibilli, L., Saint-Girons, I., Chalvignac, M. A., Le Bras, G., Cohen, G. N., & Yaniv, M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5730–5733.

Müller, K., & Garel, J. R. (1984a) *Biochemistry 23*, 655–660.

Müller, K., & Garel, J. R. (1984b) *Biochemistry 23*, 651–654.

Müller, K., & Garel, J. R. (1984c) *J. Biol. Chem. 259*, 2252–2256.

Nall, B. T., Garel, J. R., & Baldwin, R. L. (1978) *J. Mol. Biol. 118*, 317–330.

Schmid, F. X., & Baldwin, R. L. (1979) *J. Mol. Biol. 135*, 285–287.

Schulz, G. E., & Schirmer, R. H. (1979) *Principles of Protein Structure*, p 87, Springer, New York.

Shimada, K., & Szwarc, M. (1975) *J. Am. Chem. Soc. 97*, 3313–3321.

Sibilli, L., Le Bras, G., Le Bras, G., & Cohen, G. N. (1981) *J. Biol. Chem. 256*, 10228–10230.

Stellwagen, E. (1979) *J. Mol. Biol. 135*, 217–229.

Teschner, W., Rudolph, R., & Garel, J.-R. (1987) *Biochemistry* (following paper in this issue).

Vêron, M., Guillou, Y., & Cohen, G. N. (1985) *FEBS Lett. 181*, 381–384.

Von Hippel, P. H., & Wong, K. Y. (1965) *J. Biol. Chem. 240*, 3909–3923.

Wetlaufer, D. B. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 697–701.

Wetmur, J. G., & Davidson, N. (1968) *J. Mol. Biol. 31*, 349–370.

Zetina, C. R., & Goldberg, M. E. (1982) *J. Mol. Biol. 157*, 133–148.

Zettlmeissl, G., Teschner, W., Rudolph, R., Jaenicke, R., & Gade, G. (1984) *Eur. J. Biochem. 143*, 401–407.