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Independent Folding Regions in Aspartokinase–Homoserine Dehydrogenase†

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ABSTRACT: The folding of two monofunctional fragments of aspartokinase–homoserine dehydrogenase I has been studied. One of these fragments corresponds to the kinase activity and the N-terminal part of the polypeptide chain; the other one corresponds to the dehydrogenase activity and to the C-terminal part of the chain. Both fragments are able to refold into an enzymatically active conformation after complete disruption of their native structure. The kinase fragment folds up into an active monomeric species. The dehydrogenase fragment first folds up into an inactive monomeric species and then associates into an active dimeric species. These two fragments thus correspond to regions capable of autonomous folding. The folding of each of these fragments is compared to that of the corresponding region in the intact aspartokinase–homoserine dehydrogenase I reported previously [Garel, J. R., & Dautry-Varsat, A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3379–3383]. It is concluded that the N- and C-terminal regions of the intact polypeptide chain behave as independent folding units. A model of the sequence of steps involved in the folding process of aspartokinase–homoserine dehydrogenase I is presented; its relevance to the evolution of this protein is also discussed.

Two different approaches, X-ray crystallography and fragmentation studies, have shown that the tertiary structure of large polypeptide chains results from the assembly of several compact regions called domains.[1] Indeed, the structure of large proteins, as seen by X-ray crystallography, reveals the presence of compactly folded blocks, each corresponding sometimes to a continuous segment of the polypeptide chain (Liljas & Rossmann, 1974; Schulz & Schirmer, 1979). Also, it is often possible to obtain smaller folded fragments from large proteins by enzymatic or chemical cleavage of the polypeptide chain or by nonsense mutation in the corresponding gene. These fragments can be considered folded when they retain some of the properties of the protein they derive from: globular structure, ability to bind specific ligands, oligomeric state, recognition by specific antibodies, enzymatic activity, etc. (e.g., Porter, 1959; Setlow & Kornberg, 1972; Véron et al., 1972; Cohen & Holzer, 1979). Some of these properties even suggest that, when stable, this folded conformation resembles that of the corresponding segment of the chain in the folded intact protein. Besides, some of these fragments represent autonomous folding units, as judged by their capacity to resume this stable conformation after complete unfolding (Jacobson & Rosenbusch, 1976; Geisler & Weber, 1976; Högberg-Raibaud & Goldberg, 1977; Ghelis et al., 1978; Dautry-Varsat & Garel, 1978). The existence, stability, and ability to refold of these compact regions suggest that they play a role in the folding process of the entire protein. This is usually described by a sequential mechanism: the folding of a large polypeptide chain would begin with the independent folding of different segments into compact units, which would then assemble into a folded chain. In the case of an oligomeric protein, the final structure is achieved by the association between such folded chains.

We have previously investigated the folding process of a large protein, the bifunctional enzyme aspartokinase I–homoserine dehydrogenase I (AK-HDH)[2] from *Escherichia coli* K12. This enzyme is a tetramer of four identical subunits of molecular weight 89 000 (Falcoz-Kelly et al., 1972). Each chain carries the sites for both enzymatic activities, and its

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[1] We use the word region to design compactly folded parts of the polypeptide chain which have been defined functionally and keep the word domain to design such compactly folded parts defined structurally, by X-ray crystallography. Since the X-ray structure of AK-HDH is unknown, the correspondence between these two definitions cannot be made at the present time.

[2] Abbreviations used: AK-HDH, aspartokinase I–homoserine dehydrogenase I (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.

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amino acid sequence has been recently determined (Katinka et al., 1980). The kinase and dehydrogenase activities have been respectively assigned to the N-terminal and C-terminal moieties of each chain (Vêron et al., 1972). The choice of such a bifunctional enzyme allows monitoring of the folding of one or the other moiety of the protein by measuring the appearance of the activity expressed by it. A study of the kinetics of reappearance of the kinase and dehydrogenase activities during refolding of entire AK-HDH has shown that the N- and C-terminal halves of the polypeptide chain fold up independently of each other (Garel & Dautry-Varsat, 1980). In the present work, we focus on the folding of one or the other region of AK-HDH, kinase or dehydrogenase, when isolated as a smaller fragment. It is, indeed, possible to obtain two smaller, enzymatically active, monofunctional fragments from AK-HDH. The first one, the AK fragment, possesses the kinase activity and is obtained from an ochre mutant of the *thr*A gene; it is isolated as a tetramer of molecular weight 4 × 48 000 and corresponds to the N-terminal segment of AK-HDH (Sibilli et al., 1977). The second fragment, the HDH fragment, possesses the dehydrogenase activity and is obtained by limited proteolysis; it is a dimer of molecular weight 2 × 55 000 and has the C-terminal amino acid sequence of AK-HDH (Vêron et al., 1972). Here we show that, besides being stable by themselves in an active conformation, both these fragments are able to recover their enzymatic activity after complete unfolding. Then these fragments correspond to two autonomous folding units of entire AK-HDH. We also compare the kinetic mechanism of folding of each of the two regions, kinase or dehydrogenase, either when isolated in a fragment or when integrated into the whole protein. This comparison suggests that the folding process of intact AK-HDH does involve the independent folding of these two regions and supports the sequential mechanism given above.

Materials and Methods

Materials. All chemical compounds used were of analytical grade and were purchased from Sigma and Merck, except guanidine hydrochloride (Gdn-HCl) which was from Carlo Erba. The auxiliary enzymes used for the kinase coupled assay were from Sigma. DL-Asparate semialdehyde was prepared by ozonolysis of DL-allylglycine (Black & Wright, 1955). The AK fragment was purified from *E. coli* K12 strain Gif 101 (Costrejean, 1977). The HDH fragment was obtained by limited proteolysis of native AK-HDH by chymotrypsin (Vêron et al., 1972).

Methods. Enzymatic Assays. The kinase activity was measured by the coupled assay described by Wampler & Westhead (1968), and the dehydrogenase activity was tested directly (Truffa-Bachi & Cohen, 1970). Both assays were found to be linear with respect to enzyme concentration in the whole range used in this work.

Denaturation. Proteins were denatured for 30 min in 6 M Gdn-HCl, 0.1 M potassium phosphate buffer, 0.2 M KCl, and 0.01 M dithiothreitol, pH 7.2, at 50 °C and then equilibrated to 28 °C in the same medium.

Renaturation. For initiation of their refolding, the denatured fragments were diluted at least 100-fold to a final protein concentration ranging from 0.6 to 10 nM. The renaturation buffer was 0.1 M potassium phosphate, 1 M KCl, 0.01 M K_2MgEDTA, 0.01 M dithiothreitol, and 150 μg/mL bovine serum albumin, pH 7.2, at a temperature of 28 °C.

The dehydrogenase activity was measured in that same buffer just after adding the substrates NADPH and aspartate semialdehyde. For measurement of the kinase activity, samples of the AK fragment were diluted 1:3 from the renaturation buffer into the appropriate mixture for the coupled assay.

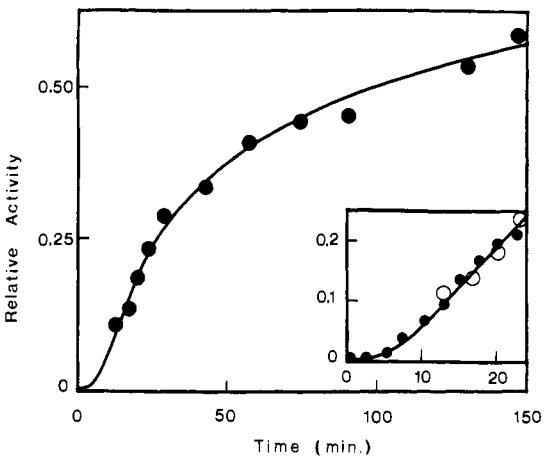

FIGURE 1: Kinetics of refolding of the HDH fragment as measured by the reappearance of its dehydrogenase activity at a final monomer concentration of 7.2 nM. Activity is expressed relative to that of the native fragment. (Inset) Enlargement of the beginning of these kinetics showing the lag phase. (●) Values obtained upon refolding the HDH fragment in the presence of its substrates, NADPH and aspartate semialdehyde; (○) points obtained as in Figure 1, i.e., to refolding in the absence of substrates.

Renaturation was followed by the reappearance of enzymatic activities as monitored from the changes in absorbance at 340 nm in a Cary 17 spectrophotometer. It was checked that the small amount of Gdn-HCl introduced into the assay when the unfolded protein is diluted does not interfere with activity measurements.

The systematic use of siliconed glassware and the presence of bovine serum albumin in renaturation experiments are essential to obtain high yields of refolding.

Results

Refolding of the AK and HDH fragments of AK-HDH has been measured by the recovery of their enzymatic activity, kinase and dehydrogenase, respectively.

Refolding of the HDH Fragment. Figure 1 gives the time course of the refolding of the HDH fragment as measured by the reappearance of its dehydrogenase activity. The activity vs. time curve shows a lag phase at the beginning of the refolding reaction. This lag phase is more obvious when the HDH fragment is renatured in the same buffer with the substrates (inset of Figure 1). There is no detectable difference of the refolding reaction of the HDH fragment when renatured in the presence or in the absence of its substrates. At later times of refolding, after this early lag phase is completed, the reappearance of dehydrogenase activity is governed by a bimolecular reaction. This is shown by the straight lines obtained in a double-reciprocal second-order plot, as well as by the concentration dependence of the slopes of these straight lines (Figure 2). The extrapolation to infinite time of renaturation kinetics shows that 80%–90% of the original activity can be recovered.

The fact that the reappearance of enzymatic activity obeys second-order kinetics indicates that it depends upon an association reaction. This association reaction yields enzymatically active species and has a rate constant of $3 \pm 1 \times 10^4$ M^{-1} s^{-1}. This association step is preceded by another reaction, which causes the early lag phase. This first reaction apparently obeys first-order kinetics with a rate constant of $1 \pm 0.5 \times 10^{-3}$ s^{-1}. It is not accompanied by the formation of active species, as judged from the low initial rate of activity regain (inset of Figure 1). Such biphasic refolding kinetics, with a first-order preceding a second-order reaction, have been observed for

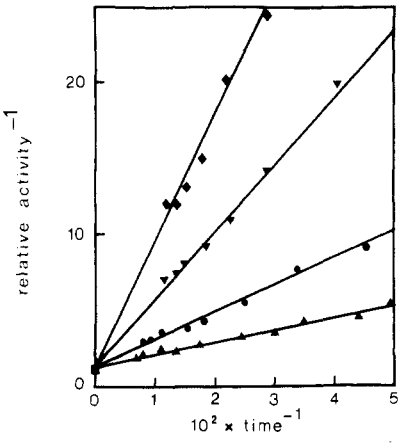

FIGURE 2: Second-order double-reciprocal plot of the kinetics of refolding of the HDH fragment as measured by its dehydrogenase activity. Final monomer concentration is 7.2 (▲), 3.6 (●), 1.5 (▼), and 0.7 nM (♦). Activity is expressed as in Figure 1: (■) corresponds to the native fragment, i.e., to a relative activity of 1. Shown in this plot are only the measurements made after 20 min, i.e., after completion of the lag phase.

several oligomeric enzymes (Engelhard et al., 1976; Rudolph et al., 1977; Dautry-Varsat & Garel, 1978; Jaenicke & Rudolph, 1980), among which there is intact AK-HDH, following its dehydrogenase activity (Garel & Dautry-Varsat, 1980). The native HDH fragment is a dimer; therefore it is likely that the association step which generates enzymatic activity is the formation of dimeric species. The first reaction, which produces the lag phase, would then be a folding step occurring within a single polypeptide chain and yielding folded monomers able to associate into active dimers. The folding of the HDH fragment follows a two-step mechanism:

$$2M_U \xrightarrow{k_1} 2M_F \xrightarrow{k_2} D$$

where M_U and M_F are the unfolded and (at least partially) folded monomers and D is the active dimer. The early lag phase corresponds to the time needed to form enough M_F from M_U so that the monomolecular step $M_U \rightarrow M_F$ is no longer rate limiting as compared to the bimolecular step $2M_F \rightarrow D$. The experimental data can fitted to the analytical solution of this two-step mechanism (Chien, 1948) to calculate a specific activity for M_F, which is at most very low, and the values of the rate constants given above $k_1 = 1 \pm 0.5 \times 10^{-3}$ s^{-1} and $k_2 = 3 \pm 1 \times 10^4$ M^{-1} s^{-1}.

Refolding of the AK Fragment. The AK fragment when kept in the presence of threonine and at high protein concentration (such as an ammonium sulfate precipitate) is tetrameric (Truffa-Bachi et al., 1974). However, removing threonine results in a rapid dissociation into monomeric species. Incubation at low protein concentrations, of the order of those used in refolding experiments, also leads to dissociation into monomers, whether threonine is present or not (Janin et al., 1967). This dissociation cannot be reversed by adding back threonine and/or concentrating the protein (Costrejean, 1977). The oligomeric state of the AK fragment seems then to be a metastable state, at least in our experimental conditions, the monomeric state being the stable one. The monomers of the AK fragment are fully enzymatically active (Janin et al., 1967; Truffa-Bachi et al., 1974).

Figure 3 shows the kinetics of reappearance of the kinase activity of the AK fragment upon refolding. The reaction is rapid and leads to the recovery of 70%–80% of the original activity. These refolding kinetics follow a single exponential, corresponding to a first-order rate constant of $3 \pm 1 \times 10^{-3}$ s^{-1} independently of protein concentration (Figure 4).

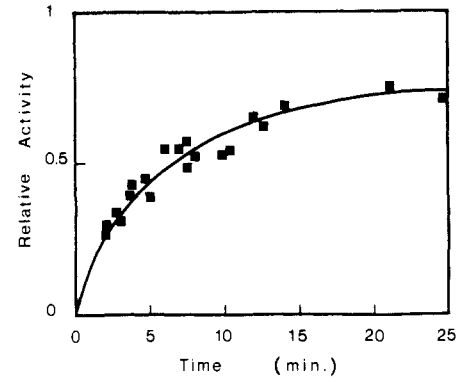

FIGURE 3: Kinetics of refolding of the AK fragment as measured by the reappearance of its kinase activity at a final monomer concentration of 10 nM. Activity is expressed relative to the native fragment.

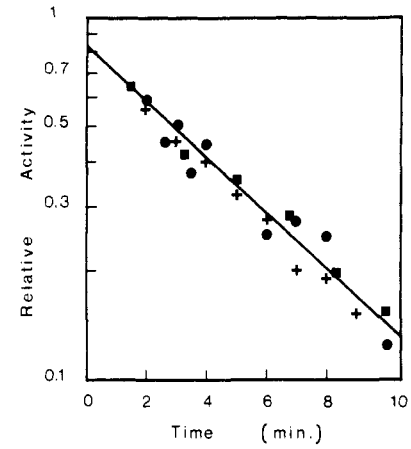

FIGURE 4: First-order semilogarithmic plot of the kinetics of refolding of the AK fragment as measured by its aspartokinase activity. Activity is expressed relative to that of the native fragment. Final monomer concentration is 10 (+), 4 (●), and 2 nM (■).

The refolding of the AK fragment obeys first-order kinetics, and this fragment when native, in our experimental conditions, is active and monomeric; this strongly suggests that the product of the refolding reaction is the AK fragment in its monomeric state. The allosteric effector of entire AK-HDH, threonine, which stabilizes the metastable tetrameric form of the AK fragment, also inhibits its kinase activity (Janin et al., 1967). The dissociation into monomers upon removal of threonine is accompanied by a complete loss of sensitivity to threonine inhibition (Janin et al., 1967). This loss cannot be reversed by the addition of threonine. In the present experiments on the refolding of the AK fragment, no recovery of the threonine inhibition of the kinase activity could be detected, even after several hours. It appears that the stable state of the AK fragment, i.e., the monomeric, enzymatically active, and threonine-insensitive form, is attained whether starting from unfolded polypeptide chains or starting from a folded tetrameric species.

Discussion

The refolding of the AK and HDH fragments and in a previous work of entire AK-HDH (Garel & Dautry-Varsat, 1980), has been followed by measuring the reappearance of enzymatic activity. Other studies of the renaturation of several proteins have shown that the reappearance of activity is a good index for the formation of the native structure (Teipel & Koshland, 1971; Engelhard et al., 1976; Jaenicke & Rudolph, 1980). Enzymatic activity is usually one of the last (if not the last) properties of the native protein to be recovered upon renaturation, indicating that it depends quite strictly on the

completion of the folding process (Koshland & Neet, 1968). Measuring enzymatic activities is not only a very convenient method but it is also very sensitive and therefore well suited for the low protein concentrations often required to obtain high yields of refolding (Jaenicke & Rudolph, 1980; Garel & Dautry-Varsat, 1980).

The Isolated AK and HDH Fragments Are Able To Refold by Themselves. Two smaller fragments, the AK and HDH fragments, can be derived from AK-HDH and are stable and functional by themselves. When isolated, they are also able to resume their native structure after complete unfolding. The complete polypeptide chain of AK-HDH is not needed for the folding of one of its parts: the N- or C-terminal moiety of this chain is able to reach its functional structure in the absence of the other moiety. Then, intact AK-HDH appears to be composed of two autonomous regions which each behave like a bona fide protein when isolated: each of them is stable, endowed with enzymatic activity, and able to refold.

The fact that the AK fragment does renature in vitro is not unexpected as this fragment comes from a nonsense mutant which uses it to grow (Janin et al., 1967). The mutant protein must therefore be able to fold up in vivo into a functional species. However, the AK fragment is purified as a tetramer whereas its stable state is the monomer (see above). Two explanations can be proposed for this discrepancy: either our experimental conditions are only a remote approximation of the intracellular conditions or the mutant protein has been synthesized in a form yielding the tetramer and then processed to give a shorter chain, the stable state of which is now the monomer. The latter explanation can be directly tested by locating the site of the nonsense mutation on the DNA and comparing it with the C-terminal end of the AK fragment.

The origin of the HDH fragment is quite different: it is obtained in vitro by the proteolytic removal of the N-terminal half of the AK-HDH chain (Vêron et al., 1972). Our experiments show that this fragment is an autonomous folding unit, although it corresponds to the C-terminal part of the original polypeptide chain, i.e., to that which is biosynthesized last. AK-HDH is thought to be the result of a gene fusion between a kinase and a dehydrogenase (Vêron et al., 1972). In this case, the C-terminal part of the chain has kept the ability to fold up by itself, which was the property of the ancestral dehydrogenase.

The AK and HDH fragments refold according to different kinetic mechanisms. The AK fragment resumes its native structure in a single first-order reaction. Folding of the polypeptide chain into a monomeric structure is sufficient to generate enzymatic activity. In the case of the HDH fragment, two successive reactions are needed to yield activity: a first-order reaction produces (partially) folded inactive monomers, which then associate into active dimers in a second-order reaction. The interaction between two polypeptide chains is thus needed for each of them to reach a functional conformation.

A Given Region, Kinase or Dehydrogenase, of AK-HDH Refolds Similarly When either Isolated in a Fragment, AK or HDH, or Integrated in the Entire AK-HDH. The two fragments AK and HDH of AK-HDH probably have, when native, a conformation resembling that of the corresponding polypeptide segment in intact AK-HDH, as judged from their specific activity per site close to that of the whole enzyme (Cohen & Dautry-Varsat, 1980). The recovery of enzymatic properties upon refolding indicates that the structure of a refolded fragment is similar to, if not identical with that of the original native fragment. Then it can be concluded that AK-HDH is composed of two autonomous folding units, which can by themselves refold into a conformation similar to the one they have in the whole native enzyme.

Table I: Rate Constants of the Various Steps of the Refolding Reactions of AK-HDH and Its Fragments

refolding of HDH fragment (s^{-1})	$1 \pm 0.5 \times 10^{-3}$	$3 \pm 1 \times 10^{4}$ M^{-1}
refolding of AK fragment (s^{-1})	$3 \pm 1 \times 10^{-3}$	
refolding of AK-HDH:[a]		
dehydrogenase activity (s^{-1})	$5 \pm 3 \times 10^{-4}$	$7 \pm 3 \times 10^{4}$ M^{-1}
kinase activity (s^{-1})	$6 \pm 3 \times 10^{-4}$	

[a] From Garel & Dautry-Varsat (1980).

Finding that fragments from a large protein correspond to autonomous folding units raises the question of whether they behave as such during the folding of the entire original polypeptide chain. If so, the folding of the entire chain begins with the independent folding of some of its segments and only later involves the interaction between the already folded segments. Two experimental approaches have been used to test the independence of the N- and C-terminal segments during refolding of entire AK-HDH. In a previous paper, we have compared the kinetics of folding of either the N-terminal (kinase) or C-terminal (dehydrogenase) region in the whole enzyme (Garel & Dautry-Varsat, 1980), and here we compare the folding of a given segment when isolated in a fragment or part of intact AK-HDH.

In both AK-HDH and its AK fragment, the folding of the kinase region is controlled by a first-order reaction, which yields an enzymatically active monomeric species. In both AK-HDH and its HDH fragment, the folding of the dehydrogenase region shows two steps, a monomolecular step producing folded inactive monomers, M_F, followed by a bimolecular step yielding active dimers. Thus the same kinetic mechanism governs the folding of a given region in both the isolated corresponding fragment and the entire protein. Table I shows that, in addition, the rate constants of these various steps are not strikingly different between the fragments and the protein. The two rate constants involved in folding the dehydrogenase region in the HDH fragment and the rate constant for folding the kinase region in the AK fragment are each within a factor of five of the corresponding constants for entire AK-HDH. Each of the two regions of AK-HDH folds up according to the same kinetic mechanism and with similar rate constants whether the other region is present or not. This similarity suggests that the two regions of AK-HDH fold up in the whole enzyme as independently as they do in the separated fragments. There does not seem to be any mutual influence between the N- and C-terminal halves of AK-HDH before each of them has already folded up.

AK-HDH Refolds by a Sequential Mechanism. From the present results and those of a previous work (Garel & Dautry-Varsat, 1980), a tentative model of the succession of events occurring during the folding of intact AK-HDH can be proposed (Figure 5). This model takes the following features into account:

(1) The kinase region folds up into an active monomer at similar rates in both the AK fragment and AK-HDH.

(2) The dehydrogenase region begins its folding by forming a partially folded and inactive monomer at the same rate in both the HDH fragment and AK-HDH.

(3) The kinase and dehydrogenase regions fold independently of each other in AK-HDH (see above).

(4) Association into dimeric species, which generates the dehydrogenase activity, occurs at similar rates in the HDH fragment and AK-HDH. This suggests that the formation

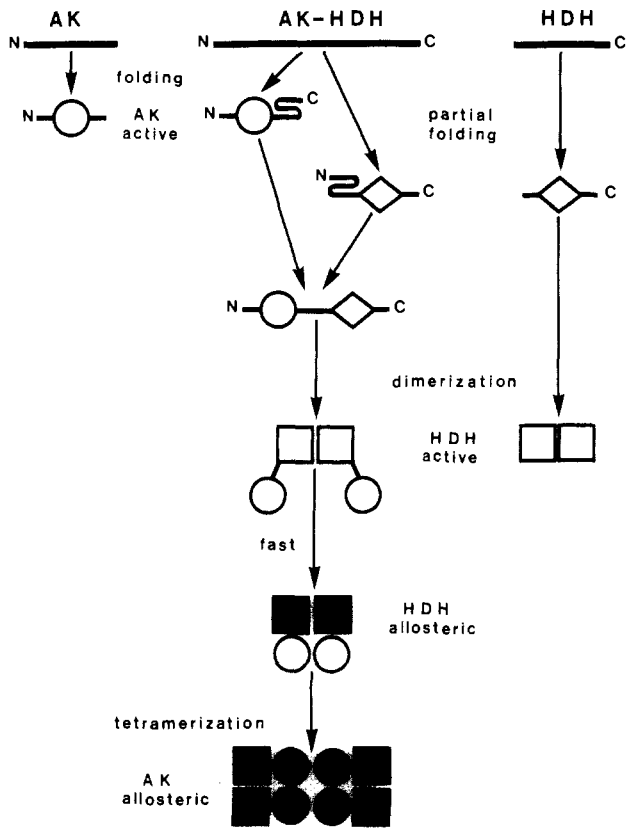

FIGURE 5: Folding pathway of AK-HDH and its fragments.

of dimers during folding of AK-HDH depends only upon the interaction between folded dehydrogenase regions.

(5) In AK-HDH, the formation of dimers leads simultaneously to the allosteric inhibition of the dehydrogenase activity, which is not the case in the HDH fragment. This indicates that the dimer of AK-HDH undergoes a fast conformational change from a nonallosteric to an allosteric state.

(6) It is the formation of tetramers which finally generates the sensitivity to threonine of the kinase activity and yields native AK-HDH. The formation of tetramers probably involves an interaction between kinase regions (Vêron et al., 1973; Cohen & Dautry-Varsat, 1980).

The folding of AK-HDH appears to be a sequential process, beginning with the independent folding of the N- and C-terminal halves of the polypeptide chain. Then these folded regions interact with each other to form the final oligomeric structure by two successive association reactions. It is striking that the three steps needed to reach a native tetramer from unfolded monomers correspond to the successive appearance of the functional properties, enzymatic activity and allosteric inhibition. In the model given in Figure 5, the appearance of enzymatic activity precedes that of the allosteric regulation of this activity, which shows that, in this case, a more elaborate structure is required for the regulation of a given activity than for its expression. This may be also true of other allosteric systems.

Folding and Evolution of AK-HDH. AK-HDH is a complex protein, with its tetrameric structure, its two activities, and its allosteric regulation. Its design by evolution has obviously needed several events, among which is gene fusion, to account for its bifunctionality (Vêron et al., 1972). Finding that the two functional units do correspond to two folding units indicates that the structures of the two fused regions of AK-HDH still resemble those of the original separated proteins. Various genetic events could have modified the structural and functional properties of the two initial partners, albeit conserving their overall conformation through evolution. It may be speculative to extrapolate the behavior of today's proteins to the processes which created them from smaller blocks. The evolution of AK-HDH, however, requires the emergence of catalytic activities, oligomeric structures, bifunctional proteins, and allosteric regulations. An evolutionary scheme must arrange these plausible events in a given order. A possibility is that the various species which appear sequentially during the folding of AK-HDH are somehow related to the succession of its proteic ancestors. Extending Haeckel's view that "ontogeny reflects phylogeny" to proteins suggests a scheme for the evolution of AK-HDH similar to that found for its folding (Figure 5). Comparable species, with the same structural and functional properties, would appear successively in the same order along the folding and evolution pathways. Thus, Figure 5 could also be a scheme for the evolution of AK-HDH.

Since the ability to fold up of each of the ancestors of a given protein had to be preserved during evolution, some features of this ancestral folding mechanism are probably conserved in this protein today. Although not as straightforward as just mentioned, there must be some correlation between folding and evolving a protein, if only because of the constraints imposed on a polypeptide chain by the necessity to fold up.

Acknowledgments

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α-Chymotrypsin Deacylation: Temperature Dependence of Hydrolysis and Transesterification Reactions†

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ABSTRACT: The hydrolysis and transesterification reactions of furoyl-chymotrypsins display nonlinear Arrhenius plots with no apparent discontinuities. Of a number of models considered, the best explanation assumes a temperature-dependent rapid equilibrium between two forms of acyl-enzyme with differing reactivities. Rate constants for the transesterification of α-chymotrypsinyl 2-(5-*n*-propyl)furoate, after normalization for this equilibrium, display a linear free energy correlation with the Taft polarity constants σ* and volumes of the attacking alcohols.

Chymotrypsin catalyzes the hydrolysis, alcoholysis, and aminolysis of a large variety of esters and amides, and the literature describing these various reactions is extensive. It is not generally appreciated, although well documented, that the temperature dependence of many chymotrypsin-catalyzed reactions is not simple, as indicated by nonlinear Arrhenius plots [Baggott & Klapper (1976) and references therein]. Nor is a complicated temperature dependence unique to this enzyme; other examples are urease (Kistiakowsky & Lumry, 1949), the citrate-condensing enzyme (Kosicki & Srere, 1961), glycogen phosphorylase (Helmrich & Cori, 1964; Graves et al., 1965), pyruvate kinase (Kayne & Suelter, 1965), D-amino acid oxidase (Massey et al., 1966), lysozyme (Saint-Blancard et al., 1977), and ribonuclease (Matheson & Scheraga, 1979). Such nonlinearity may be more common than suggested by this short list, since reliable temperature dependencies have not been reported for many enzymes.

There are four general explanations for a nonlinear Arrhenius plot: (i) the activation enthalpy is temperature dependent; (ii) the reaction proceeds through at least two parallel paths to the same product (i.e., different reaction mechanisms are possible), with pathway predominance dependent on temperature; (iii) there is one (or more) kinetically significant reaction intermediate with a temperature-dependent shift in rate-limiting step; or (iv) there is a temperature-dependent equilibrium between enzyme forms which differ in catalytic efficiency. Since each of these general explanations may subsume two or more specific reaction mechanisms, or a combination from these four possibilities may be involved, a nonlinear Arrhenius plot introduces interpretive complications. In the past, an equilibrium between enzyme forms has generally been posited in explanation (e.g., Rajender et al., 1970; Wedler et al., 1975), and in the case of chymotrypsin there is evidence to support such a hypothesis. The binding of inhibitors and substrates to α-chymotrypsin and to covalently modified forms of this enzyme show distinct deviations from linearity in van't Hoff plots (Schultz et al., 1977, 1979), and a structural transition of α-chymotrypsin under experimental conditions similar to those used in our own kinetic studies (Baggott & Klapper, 1976) has been reported (Kim & Lumry, 1971; Lumry & Biltonen, 1979). Nonetheless, there is reason to consider the remaining three models as well. From our general knowledge of chemical reactions it is unlikely that $A\!H^{\ddagger}$ would be temperature independent, although apparent independence may hold over the small temperature range to which most enzyme studies are limited. On the basis of the proton inventory technique (Schowen, 1977) the serine proteases may be capable of catalyzing reactions by more than one pathway, involving either a single proton or a cooperative multiple proton mechanism. Finally, a tetrahedral reaction intermediate has been commonly assumed as an obligatory step in serine protease catalysis (Bender & Kilheffer, 1973). O'Leary & Kluetz (1972) have proposed a pH-dependent change in rate-limiting

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