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Refolding of a bifunctional enzyme and its monofunctional fragment

(protein folding/protein domains/limited proteolysis/aspartokinase II-homoserine dehydrogenase II/kinetics)

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ABSTRACT **The renaturation of the bifunctional enzyme aspartokinase II-homoserine dehydrogenase II has been studied by using the reappearance of its two activities. The same kinetics of renaturation are obtained for the dehydrogenase (EC 1.1.1.3) and the kinase activity (EC 2.7.2.4). The mechanism of refolding of the enzyme apparently involves two steps, a folding step occurring within a monomer and a subsequent dimerization step. The reappearance of the two activities depends on this dimerization step, suggesting that monomeric species are inactive. A proteolytic fragment possessing full dehydrogenase activity is shown to be able to renature, as judged by the recovery of its activity. In this case also, the refolding depends on the formation of dimeric species. However, the refolding of this fragment is much faster than that of the dehydrogenase region in the intact enzyme. These results suggest that, although the dehydrogenase region can refold by itself when isolated as a fragment, refolding of this same region in the whole protein involves interactions with the remainder of the protein.**

Large and complex proteins are often oligomeric structures in which several polypeptide chains are associated. Also, in each subunit, the polypeptide chain is often folded up into several compact regions called domains (1, 2). Specific intra- or interchain interactions between the various domains of the protein are involved in both the tertiary structure of each subunit and the quaternary structure of the oligomer. Beside x-ray crystallography, one usual way of finding evidence for domains in a large protein is to obtain smaller fragments still possessing a tightly folded structure and sometimes even endowed with a partial activity (3–8). The comparison between the properties of the same domain(s) as either part of the whole assembly or as an isolated fragment can be used to probe the existence and role of some of the interactions involving this particular domain.

The native conformation of a large protein, as represented by its different domains and the set of interactions between them, has to form during the folding process from the unfolded and separated chains. Study of the folding mechanism may then reveal an order in the formation and assembly of the different domains that will finally yield the native protein. In such a study, monomolecular processes occurring within a chain and multimolecular processes implying several chains can be experimentally distinguished because of their different concentration dependences. Also, a comparative study of the folding mechanisms of the entire protein and of one of its smaller fragments may reveal an important role of some of the interdomain interactions, not only in the finally assembled molecule but also while it is forming. The preceding considerations explain why folding studies of large proteins, and when possible of their smaller fragments, could be a tool for understanding some of the properties of their native conformation.

The enzyme aspartokinase II-homoserine dehydrogenase II (AK II-HDH II) from *Escherichia coli* K-12 appears to be well suited for this type of study. It is a dimer made of identical subunits ($M_r = 2 \times 88{,}000$) that catalyzes two different reactions: the conversion of aspartate into β-aspartyl phosphate (kinase activity, EC 2.7.2.4) and the conversion of aspartate semialdehyde into homoserine (dehydrogenase activity, EC 1.1.1.3) (9, 10). Limited proteolysis of AK II-HDH II yields two fragments, both of them dimeric (11). The larger fragment ($M_r = 2 \times 37{,}000$) is endowed with full dehydrogenase activity, which shows that its conformation is close to that of the same region in the whole protein. The smaller fragment ($M_r = 2 \times 24{,}000$), although devoid of enzymatic activity, still possesses a folded structure, as seen from its dimeric state. AK II-HDH II appears then to be composed of (at least) two compact regions, one of which can be isolated as such in the active HDH II fragment.

This article shows that indeed AK II-HDH II can be completely renatured from its unfolded and separated chains, as seen from the recovery of both enzymatic activities. Kinase and dehydrogenase activities reappear at the same rate, mainly governed by a bimolecular reaction. This indicates that formation of the native structure of AK II-HDH II is linked to the build-up of dimeric species, and suggests that the monomer is enzymatically inactive. The HDH II proteolytic fragment is also able to recover its dehydrogenase activity completely after unfolding, which shows that the part of the polypeptide chain corresponding to this fragment represents an independent folding unit. In this case also, formation of the fragment native structure depends upon a bimolecular association step. However, the rate of formation of native HDH II fragment is much higher than that of native AK II-HDH II, which suggests that the kinase and dehydrogenase regions of the whole enzyme do not behave independently during the folding process, and maybe not in the folded native protein either.

EXPERIMENTAL

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 aspartokinase coupled assay were from Sigma. DL-Aspartate semialdehyde was prepared by ozonolysis of DL-allylglycine (12).

AK II-HDH II and its HDH II proteolytic fragment were purified and characterized as described previously (10, 11, 13). Protein concentrations were measured spectrophotometrically, using the extinction coefficient determined previously (10, 11).

Methods. *Enzymatic assays.* Aspartokinase activity was measured by the coupled assay described by Wampler and Westhead (14), and homoserine dehydrogenase activity was tested directly (15). Both assays were found to be linear with respect to enzyme concentration in the whole range used in the present work.

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Abbreviations: AK II-HDH II, aspartokinase II-homoserine dehydrogenase II; HDH II, proteolytic fragment of AK II-HDH II; Gdn·HCl, guanidine hydrochloride.

Denaturation. Proteins were denatured for 1 hr in 5.3–6 M Gdn·HCl/0.1 M potassium phosphate buffer, pH 7.2/0.2 M KCl/0.01 M dithiothreitol, 50°C, and then equilibrated at 20°C.

Renaturation. In order to initiate their renaturation, the unfolded proteins were diluted at least 1:40, to a final protein concentration ranging from 0.5 to 10 μg/ml for AK II-HDH II and from 0.1 to 1 μg/ml for its active fragment. The renaturation buffer was 0.1 M potassium phosphate/1 M KCl/0.01 M K_2MgEDTA, pH 7.2, and the temperature was 20°C.

The dehydrogenase activity was measured in the same buffer just after the substrates, NADPH and aspartate semialdehyde, were added. For the kinase activity, protein samples were diluted 1:3 from the renaturation buffer into the appropriate mixture for the coupled assay (14).

Renaturation was followed by the reappearance of enzymatic activities as monitored from the changes in absorbance at 340 nm in a Cary 17 spectrophotometer. Activities were expressed relative to that of the same concentration of native protein.

It was found that the small amount of Gdn·HCl introduced into the assay by the dilution of the unfolded proteins did not interefere with activity measurements; also, it did not have any influence on the time course of renaturation.

Using silanized glassware (Siliclad, Clay Adams, Parsippany, NJ) to avoid losses of protein due to adsorption on glass surfaces was found to be an essential factor.

RESULTS

Refolding of Intact AK II-HDH II as Measured by the Reappearance of Homoserine Dehydrogenase Activity. Fig. 1 shows the time course of reappearance of dehydrogenase activity upon renaturation of AK II-HDH II. At all enzyme concentrations studied, the activity vs. time curve shows an early lag phase. At longer times—i.e., after about 30 min—the reappearance of activity is governed by a bimolecular reaction, as seen from the dependence of its rate on the protein concentration (not shown). This bimolecular reaction can be illustrated by the straight line obtained in a double-reciprocal second-order plot (*inset* of Fig. 1). The activity that would be obtained after an infinite time of renaturation can be estimated from extrapolation of such plots, and is found very similar to that of the native enzyme at the same concentration. This shows that the maximum yield of renaturation of AK II-HDH II is not far from 100%. However, to approach such a high yield would require more than 20 hr or so, and the native enzyme itself is not stable enough at such low concentrations. The bimolecular kinetics of the reappearance of activity indicate that the rate-limiting step is the reassociation of two chains to form a dimer. From Fig. 1 and similar data, the second-order association rate constant is estimated to be on the order of 10^3 M^{-1} sec^{-1}.

The presence of an early lag phase shows that another reaction takes place prior to the formation of active dimeric species and is limiting for the appearance of species capable of reassociating. This lag phase does not depend significantly on protein concentration, which indicates that this first reaction probably takes place in a monomer. This reaction, as evidenced by the lag phase, may correspond to some kind of (partial) folding of the monomer, which results in the formation of species able to yield active dimers upon a simple bimolecular reaction.

Refolding of Intact AK II-HDH II as Measured by the Reappearance of Aspartokinase Activity. Renaturation of entire AK II-HDH II can also be measured by the reappearance of aspartokinase activity. Fig. 2 shows that the kinetics of recovery of the kinase activity are the same as those for the recovery of the dehydrogenase activity. In both cases, the same lag phase and concentration dependence in the renaturation of AK II-HDH II are observed, which shows that the reappearance of the two activities is governed by the same mechanism.

Refolding of the HDH II Fragment as Measured by the Reappearance of its Homoserine Dehydrogenase Activity. The proteolytic HDH II fragment of AK II-HDH II, endowed with full homoserine dehydrogenase activity, is also capable of renaturing, as seen from the reappearance of its enzymatic activity (Fig. 3). The kinetics of reappearance of the activity of the HDH II fragment was analyzed as described above for the entire enzyme. The result showed that (*i*) the maximum yield of renaturation is close to 100%; (*ii*) this renaturation is governed by a bimolecular reaction, with an association rate constant on the order of 2×10^4 M^{-1} sec^{-1}; and (*iii*) any reaction preceding the reassociation step should be completed in less than 2 min, as suggested by the absence of a lag phase (Fig. 3). Then, as in the case of intact AK II-HDH II, the activity reappearance of the HDH II fragment seems to depend on the formation of dimeric species. It is worth noting, however, that, if it exists, the reaction responsible for the lag phase and attributed to some partial folding of the monomer in entire AK

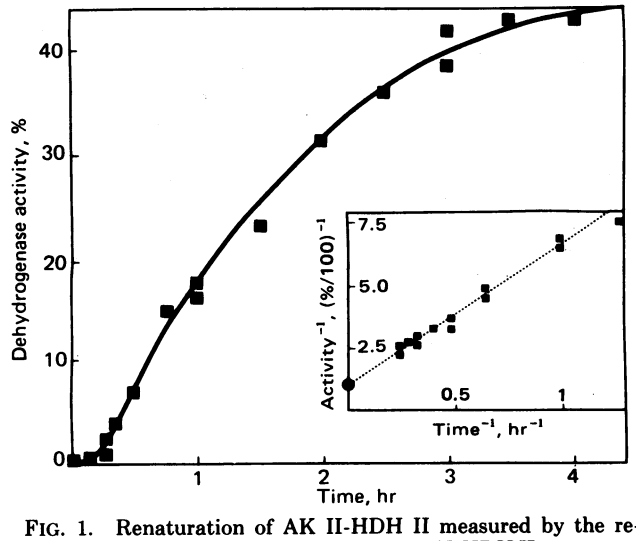

FIG. 1. Renaturation of AK II-HDH II measured by the reappearance of its dehydrogenase activity. AK II-HDH II concentration was 27 nM. (*Inset*) Second-order representation of these data. ●, Activity measured for the native enzyme at the same concentration.

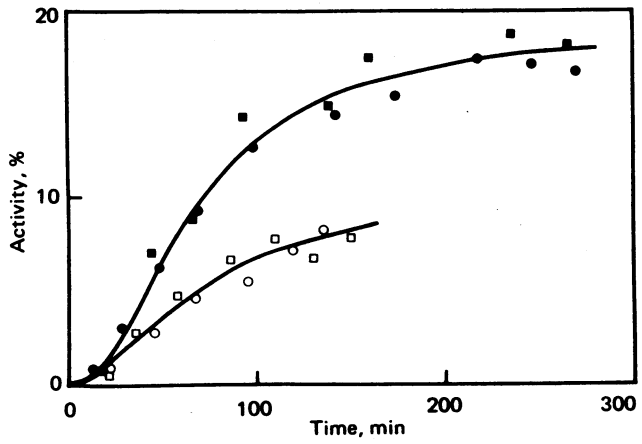

FIG. 2. Renaturation of AK II-HDH II measured by the reappearance of kinase (■, □) and dehydrogenase (●, ○) activities. AK II-HDH II concentration was 18 nM for the upper curve and 9 nM for the lower curve.

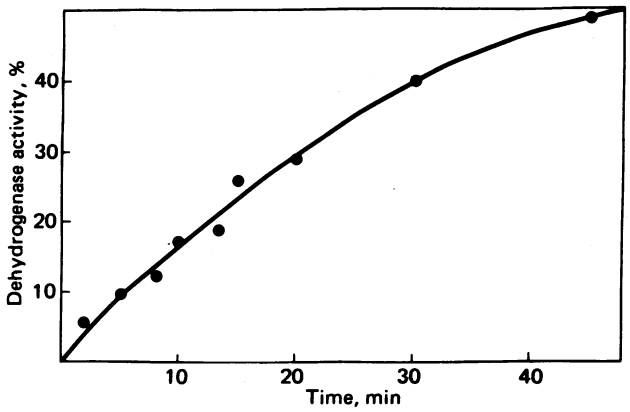

FIG. 3. Renaturation of the proteolytic HDH II fragment measured by the reappearance of its dehydrogenase activity. HDH II fragment concentration was 4 nM.

II-HDH II should be markedly faster for the HDH II fragment, for which no such lag phase is detected. Also, the rate of association into dimeric species is about 20 times higher for the HDH II fragment than for the intact enzyme.

DISCUSSION

The renaturation reactions of intact AK II-HDH II and its HDH II proteolytic fragment have been studied by measuring the kinetics of reappearance of enzymatic activity. Other studies of the renaturation of several complex enzymes, as measured by both enzymatic and physical methods, show that the reappearance of activity is a valid index for the formation of the native structure (16–19). In the case of a bifunctional enzyme, such as AK II-HDH II, it can thus be assumed that the reappearance of either activity corresponds to the formation of the native structure in that part of the molecule responsible for this activity.

AK II-HDH II and its HDH II fragment are both dimers in their native state. Except at early times, the reappearances of both the kinase and dehydrogenase activities of AK II-HDH II follow bimolecular kinetics, and thus depend on the formation of dimeric species. The presence of a lag phase at early times of renaturation shows that another reaction limits the formation of species able to reassociate into active dimers, presumably involving some partial folding of the monomer. The overall mechanism describing the refolding of AK II-HDH II is then

$$2U \rightleftharpoons 2F \rightleftharpoons D$$

in which U and F are, respectively, the unfolded and (at least partially) folded states of the monomer, and D is the native dimer. The lag phase would correspond to the time required to form enough F from U, so that the monomolecular reaction $U \rightarrow F$ is no longer rate-limiting as compared to the bimolecular reaction $2F \rightarrow D$. A similar mechanism has already been found applicable to the refolding of other oligomeric enzymes (19–21). The interesting feature of AK II-HDH II is that the same steps are apparently involved in the formation of native structure in the regions of the protein responsible for the two different activities. A more detailed kinetic analysis, according to Chien (22), of the above mechanism indicates that monomer species have no significant activity. This conclusion is in agreement with our observation that renaturing AK II-HDH II at a very low final concentration (below 5 nM) produces a mixture of monomeric and dimeric species, in which the only active species would be the dimer. Then, although there is evidently a folding step of the monomer in the refolding process of AK II-HDH II, the complete folding of each chain is achieved only upon formation of the quaternary structure.

The proteolytic HDH II fragment is not only endowed with a full dehydrogenase activity but also can resume its native structure after complete unfolding. Thus the compact region corresponding to this fragment represents a true independent folding unit. As for the entire enzyme, the reappearance of the activity of the HDH II fragment (i.e., presumably of its native conformation) depends on the formation of dimeric species. In this case, the formation of native dehydrogenase regions depends only on the intersubunit interactions existing in the HDH II fragment (i.e., those due to the dehydrogenase regions themselves).

Because the HDH II fragment possesses a full dehydrogenase activity, its conformation is likely to be very close to that of the corresponding region in intact AK II-HDH II. The fact that the HDH II fragment is by itself able to resume its native structure after unfolding raises the question of whether this fragment also represents a valid model for the dehydrogenase region of AK II-HDH II during the refolding process. If this were the case, one would expect the kinetics (monomer folding and dimerization) of the reappearance of the native dehydrogenase region in AK II-HDH II and in the HDH II fragment to be comparable. However, this does not seem to be the case. For the HDH II fragment, the folding of a monomer into a species able to dimerize is much faster than for intact AK II-HDH II, as seen from the presence of a lag phase in the latter (Fig. 1) and not in the former (Fig. 3). Also, the monomers of the HDH II fragment reassociate into dimers about 20 times faster than the monomers of AK II-HDH II. These differences indicate that the refolding of the HDH II fragment cannot be strictly the same as that of the dehydrogenase region in the whole enzyme. This conclusion is further supported by the fact that, in intact AK II-HDH II, the kinase and dehydrogenase activities reappear with the same kinetics. This suggests that common processes limit the rate of formation of the native structure in both the kinase and dehydrogenase regions of the enzyme. These processes probably involve both regions of the chain, and therefore may not be adequately represented by events occurring in the HDH II fragment. Therefore it seems that, although the HDH II fragment is an independent refolding entity and represents an intermediate state for the folding of the polypeptide chain, this fragment does not correspond to an actual intermediate in the refolding of the whole enzyme. In other words, it seems unlikely that refolding of AK II-HDH II involves intermediates with a folded dehydrogenase region and an unfolded kinase region. If such intermediates existed, even transiently, they could give rise to the formation of hybrid molecules between a complete and a partial chain interacting by the dehydrogenase region. Attempts to find evidence for such hybrid molecules were unsuccessful, in agreement with the above conclusion. The simplest explanation for the differences between the enzyme and its fragment is that an interaction between the kinase and dehydrogenase regions exists during the refolding of the former (and not of the latter), and maybe the interaction persists in native AK II-HDH II.

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