Intermediates on the Folding Pathway of Octopine Dehydrogenase from *Pecten jacobaeus*†

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ABSTRACT: Octopine dehydrogenase from *Pecten jacobaeus* is a monomeric enzyme (M_r 45 000) that catalyzes the reductive condensation of pyruvate and L-arginine to D-octopine with NAD as a cofactor. Three processes can be observed during the renaturation of this protein, corresponding to different time courses. First, a fast reaction is detected by fluorescence and circular dichroism, which results in the formation of a structured intermediate. Second, this already folded intermediate rearranges its conformation in a slower step and becomes insensitive to a short exposure to proteases. The appearance of the resistance to cleavage by trypsin or thermolysin is characterized by an activation energy of 82 kJ/mol and is not influenced by the presence of glycerol in the renaturation buffer. It is possible that this reaction is controlled by the cis–trans isomerization of prolines. Finally, the enzymatic activity is regained during the slowest step. The rate of this slow reactivation reaction is strongly dependent upon the solvent viscosity, suggesting that it involves movements of folded parts of the protein relative to each other. This step could correspond to the correct pairing between the NAD-binding domain and the substrate-binding domain, which is a prerequisite for enzyme activity. Domain pairing reactions do not occur in small proteins but are probably important events in the folding process of long polypeptide chains. In the case of octopine dehydrogenase, the domain pairing reaction is slow and hence constitutes the rate-limiting step of renaturation, thus masking the cis–trans isomerization of prolines. The slow pairing of folded domains may possibly represent a general feature of the folding process of multidomain proteins.

Kinetic studies of folding and unfolding have generally focused on small single-domain proteins of about 150 amino acid residues like ribonuclease A or lysozyme (Kim & Baldwin, 1982; Kuwajima & Schmid, 1984). The refolding of these proteins is a relatively fast process, at least under conditions that effectively stabilize their native state. The slowest reaction, which controls the rate of formation of the fully native protein, appears to be related to the cis–trans isomerization of X–Pro bonds of the polypeptide chain. Small proteins, however, represent a rather limited fraction of the known proteins. Many proteins are oligomers and often composed of chains that are longer than 150 residues. Reconstitution studies with a number of oligomeric proteins having chains longer than 300 residues show the presence of very slow folding steps, which cannot be accounted for by proline isomerization (Zettlmeissl et al., 1982; Rudolph et al., 1986). The renaturation of these oligomeric proteins from their unfolded and separated chains implies a mixture of folding and association reactions (Jaenicke, 1982, 1984). In these cases, the specific analysis of the folding steps within one chain is complicated owing to the subsequent fast and slow association reactions (Jaenicke & Rudolph, 1983, 1986). An oligomeric protein is therefore not the most convenient system for studying the formation of the native structure in a large polypeptide chain.

Octopine dehydrogenase from *Pecten jacobaeus* is a large monomeric enzyme with a relative molecular mass of 45 000 (Zettlmeissl et al., 1984). It is thus a suitable polypeptide for the analysis of the slow structural rearrangements as well as for the characterization of structured intermediates that are involved in the refolding of a single chain. After complete denaturation in 6 M guanidine hydrochloride, refolding of ODH[1] starts with a fast reaction as seen from changes in protein fluorescence and far-UV circular dichroism. The reactivation of the enzyme occurs in a second slow reaction (Zettlmeissl et al., 1984). This slow reaction takes place within a folded state and could involve movements of different parts of the chain relative to each other. If so, the rate of this reaction should be dependent on the viscosity of the solvent. The viscosity dependence of a rate constant can indicate that the corresponding process is hydrodynamically limited. It is the viscosity dependence of the rate of renaturation of double-stranded DNA that suggests that the friction of the coiled strands on the solvent is the rate-controlling factor (Wetmur & Davidson, 1968). To modify the viscosity of the solvent during refolding of octopine dehydrogenase, glucose, glycerol, poly(ethylene glycol) (PEG) or poly(vinylpyrrolidone) (PVP) is added to the renaturation buffer. These additives can have two effects on protein folding: (i) modifying the stability of the native state or that of an intermediate structure and/or (ii) increasing the viscosity of the solvent. In the case of ODH, using different solvent additives can help to separate their effects on solvent viscosity from their effects on protein structure.

The kinetics of renaturation of ODH have also been measured by the disappearance of the susceptibility to proteolytic cleavage. Two proteases with different specificities, trypsin

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[1] Abbreviations: ODH, octopine dehydrogenase from *Pecten jacobaeus* (EC 1.5.1.11); EDTA, (ethylenedinitrilo)tetraacetic acid; GdnHCl, guanidine hydrochloride; PEG, poly(ethylene glycol); PVP, poly(vinylpyrrolidone).

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and thermolysin, were used to examine the folding pathway of ODH. Protease pulse experiments show that some sites which are still accessible after the rapid initial folding become resistant to cleavage prior to the final slow reactivation step. The rate at which ODH becomes resistant to proteases is not affected by the presence of glycerol.

Three different processes can be observed during the renaturation of ODH. A fast process involves the formation of a structured intermediate. A slower process is monitored by the recovery of the resistance to proteases. The reappearance of enzymatic activity is the slowest process, and its rate depends on the solvent viscosity.

Experimental Procedures

Materials

NADH (Boehringer, Mannheim), L-arginine (Serva), and pyruvate (Merck, Darmstadt) were used for activity measurements. Glycerol (87%), poly(ethylene glycol) 400 (for gas chromatography), and glucose were supplied by Merck (Darmstadt), and poly(vinylpyrrolidone) K30 was supplied by Fluka (Buchs). Trypsin (TPCK-treated) and soybean trypsin inhibitor were purchased from Sigma Chemical Co. (München). Thermolysin was obtained from Boehringer (Mannheim), urea (99.5%) from Roth, and Gdn·HCl from Schwarz/Mann (Orangeburg, NY). Two forms of octopine dehydrogenase, A and B, are found in the adductor muscle of *Pecten jacobaeus*. Except for the electrophoretic mobility, both forms of the enzyme exhibit identical physicochemical, enzymatic, and folding properties (Zettlmeissl et al., 1984). In this investigation the B form of ODH was used. It was isolated as previously described from animals containing exclusively this type of the enzyme (Zettlmeissl et al., 1984). All other reagents were of A grade from Merck (Darmstadt). Quartz-bidistilled water was used throughout.

Methods

Reactivation in the Presence of Various Additives in the Refolding Buffer. Denaturation of ODH was achieved by 1:4 dilution of the stock solution (protein concentration 1 mg/mL) with 8 M Gdn·HCl in 0.1 M sodium phosphate, pH 7.6, plus 1 mM dithioerythritol and 1 mM EDTA so that the final Gdn·HCl concentration was 6 M. Renaturation was carried out by a 1:60 dilution of the denatured ODH into a buffer containing 0.1 M sodium phosphate, 1 mM dithioerythritol, and 1 mM EDTA at pH 7.6. Glycerol, glucose, poly(ethylene glycol) 400, or poly(vinylpyrrolidone) was added to the renaturation buffer in order to change its viscosity. In the case of glycerol the increase in viscosity was taken from tabulated values (*Handbook of Chemistry and Physics*, 1974). The viscosities of the solutions containing the other additives were measured in a capillary viscosimeter calibrated with glycerol solutions of known viscosities. It was verified that none of the additives present during renaturation interfered with the enzymatic assay.

Competition between Refolding and Proteolysis. Native ODH is resistant to inactivation by proteolytic cleavage: at 20 °C its activity remains unchanged after 24 h of incubation with up to 4 μg/mL trypsin or 10 μg/mL thermolysin. Under the same conditions unfolded ODH and/or early folding intermediates are rather sensitive to proteolysis. This can be shown by competition experiments between refolding and cleavage performed as follows. ODH was unfolded for 1 h by 8 M urea at pH 3. Renaturation and proteolysis were initiated at the same time by 1:80 dilution of the denatured enzyme in 0.1 M sodium phosphate, 1 mM dithioerythritol, and 1 mM EDTA, at pH 7.6 and in the presence of increasing concentrations of trypsin. After variable intervals of time, the proteolytic reaction was stopped by addition of soybean trypsin inhibitor (at a final concentration twice that of trypsin) while the refolding reaction was allowed to proceed further for 60 min (this time is sufficient for the complete reactivation of uncleaved ODH). The fraction of intact ODH was then determined by the recovery of enzymatic activity. Figure 1a shows the result of such an experiment: the presence of 4 μg/mL trypsin during the first 2 min of renaturation is sufficient to cleave all the ODH molecules as judged from their inability to regain their activity. Similar experiments were performed with thermolysin. In this case, the renaturation mixture contained only 2.5 μM EDTA, and the cleavage reaction was stopped by addition of EDTA to a final concentration of 22.5 mM. Figure 1b shows that 10 μg/mL thermolysin present during the first 2 min of renaturation is sufficient to cleave all ODH molecules and prevent them from reactivation. These results indicate that the resistance to either protease is recovered rather slowly during refolding of ODH.

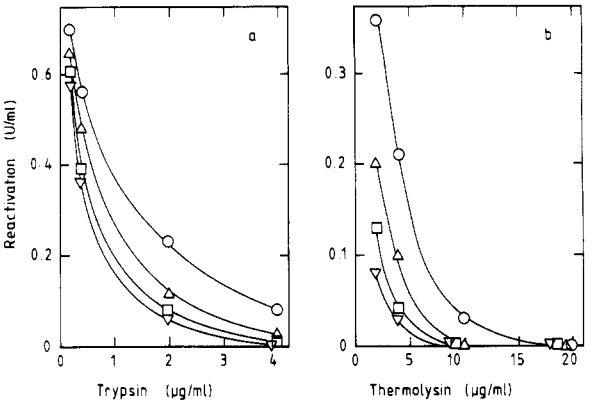


FIGURE 1: Competition between refolding of ODH and proteolytic cleavage by (a) trypsin or (b) thermolysin. Denaturation, renaturation, and proteolysis are as described under Methods. Trypsin or thermolysin was inactivated after 30 (○), 60 (△), 90 (□), and 120 s (▽) by the addition of soybean trypsin inhibitor or EDTA, respectively.

Protease Pulse Experiments during Renaturation of ODH. ODH was submitted to short pulses of proteolysis by trypsin or thermolysin at various times during its refolding. Renaturation was initiated by a 1:80 dilution of urea-denatured ODH into 0.1 M sodium phosphate, 1 mM dithioerythritol, and 1 mM EDTA, pH 7.6 at 20 °C. After variable intervals of time, trypsin in the same buffer was added to a final concentration of 4 μg/mL, and proteolysis was allowed to proceed for 2 min. Soybean trypsin inhibitor in the same buffer was then added in a 2-fold molar excess to the trypsin concentration. The residual ODH activity was measured after 24 h. The same experiment was also carried out with glycerol being present in the renaturation buffer. When the trypsin pulse experiments were performed at a lower temperature (10 or 15 °C instead of 20 °C), a higher concentration of trypsin was used (12.5 instead of 4 μg/mL) in order to guarantee cleavage of all the susceptible ODH molecules.

Protease pulse experiments with thermolysin were performed as described for trypsin measurements except for the following modifications: the renaturation mixture contained only 2.5 μM EDTA; complete cleavage of all susceptible polypeptide chains was achieved by a pulse of 2 min with 10 μg/mL thermolysin after which the protease was inactivated by the addition of EDTA to a final concentration of 22.5 mM.

Results

Refolding of ODH in the Presence of Glucose, Glycerol, Poly(ethylene glycol), or Poly(vinylpyrrolidone). In the ab-

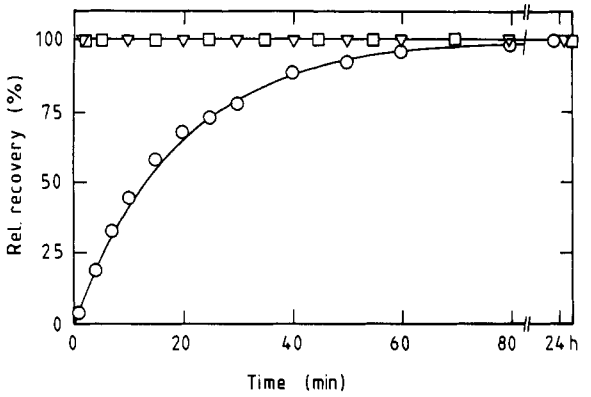


FIGURE 2: Refolding and reactivation of ODH after 1-h denaturation in 6 M Gdn·HCl at 20 °C as determined by protein fluorescence at 330 nm (□), relative ellipticity at 222 nm (▽), and the regain of activity (○). The respective data of the renatured protein were taken as 100%. Refolding was performed at 20 °C in 0.1 M sodium phosphate, 1 mM dithioerythritol, and 0.1 M residual Gdn·HCl at pH 7.6, at a protein concentration of 4.5 μg/mL.

sence of solvent additives, the refolding of ODH after long-term denaturation by 6 M Gdn·HCl is biphasic. A first rapid phase is observed by the changes in protein fluorescence (when excited at 280 nm) and in far-UV circular dichroism at 222 nm. This reaction is complete within the time required for manual mixing (≤15 s) (Figure 2), and this holds even at low temperatures (down to 5 °C) and in the presence of glycerol [up to 40% (v/v)]. The species formed in this first step is enzymatically inactive although it is largely folded, as judged by fluorescence and ellipticity. Reactivation takes place subsequently in a slow reaction with a rate constant of 9.0×10^{-4} s^{-1} at 20 °C (Figure 2).

The presence of increasing amounts of glucose (Figure 3a) or glycerol (Figure 3b) leads to a marked decrease in the rate of reactivation. In simple cases, the friction of the solvent is proportional to viscosity. Therefore, the rate of a reaction controlled by hydrodynamic friction should be inversely proportional to solvent viscosity. This appears to be true for the rate of reactivation of ODH, although slightly different slopes are observed for glucose and glycerol (Figure 4a).

Upon renaturation of ODH, only about 70% of the protein refolds to the native state. As shown by gel filtration experiments, the remaining 30% do not form aggregates as in the case of other oligomeric dehydrogenases (Jaenicke, 1982; Zettlmeissl et al., 1982). Instead, for about 30% of the ODH molecules, the first steps in refolding seem to lead to an incorrect monomeric structure that cannot proceed further to the native state. The origin of this incorrect and abortive folding is not known yet. The 30% of the nonnative ODH molecules can be denatured by 6 M Gdn·HCl and renatured again by dilution. The yield of such a "recycling" experiment again is 70%, showing that this partial reactivation is not due to an irreversible process.

The presence of these 30% incorrectly folded chains explains the slight increase in the fluorescence emitted at 350–360 nm by the reconstituted ODH as compared with the initial native protein (Figure 5). Clearly, the tryptophan residues are more exposed to solvent in these incorrectly folded chains than in the native state. Furthermore, the ellipticity at 222 nm of the reconstituted enzyme is only 70–80% of that of the original material.

The incorrectly folded molecules are formed to the same extent independent of the presence or absence of glucose or glycerol in the renaturation buffer (Figure 4b). Thus, only the rate and not the extent of ODH renaturation is affected by glucose or glycerol.

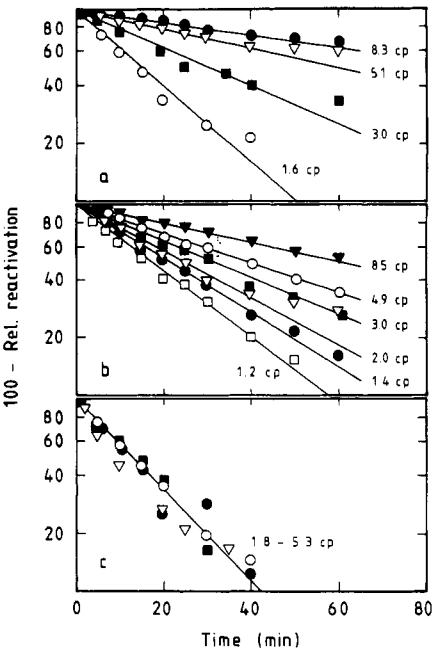


FIGURE 3: Viscosity dependence of the kinetics of reactivation of ODH in the presence of (a) glucose, (b) glycerol, or (c) poly(ethylene glycol). Denaturation by 1-h incubation in 6 M Gdn·HCl at 20 °C. Renaturation (20 °C, protein concentration 4.5 μg/mL) in 0.1 M sodium phosphate, 1 mM dithioerythritol, and 0.1 M residual Gdn·HCl at pH 7.6. The bulk viscosities of the refolding mixtures (in cP) are included in the figures. The rate constants, as determined by the given semilogarithmic linearization, are as follows: (a) (○) 7.7×10^{-4} s^{-1}, (■) 3.7×10^{-4} s^{-1}, (▽) 1.9×10^{-4} s^{-1}, and (●) 1.2×10^{-4} s^{-1}; (b) (□) 6.7×10^{-4} s^{-1}, (●) 5.4×10^{-4} s^{-1}, (▽) 4.7×10^{-4} s^{-1}, (■) 3.7×10^{-4} s^{-1}, (○) 2.9×10^{-4} s^{-1}, and (▼) 1.9×10^{-4} s^{-1}; (c) (○, ■, ▽, ●) 9.0×10^{-4} s^{-1}.

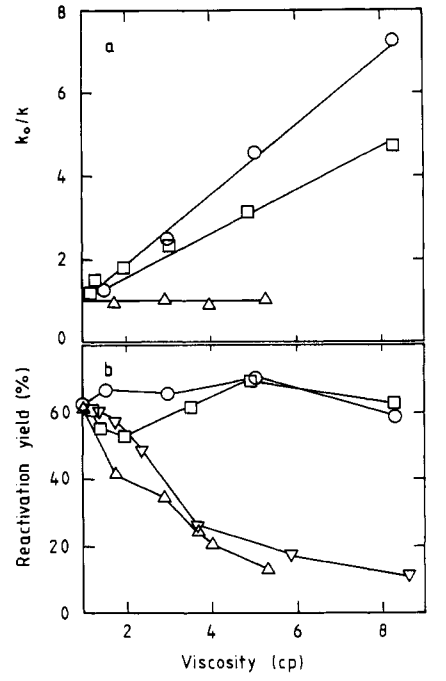


FIGURE 4: Dependence on solvent viscosity of the rate (a) and yield (b) of reactivation of ODH. The bulk viscosity of the solvent is varied by increasing the fraction of either glucose (○), glycerol (□), PEG (△), or PVP (▽). Experimental details were as in Figure 3.

In contrast to these experiments, the presence of solvent additives with a high molecular weight like PEG or PVP has a strong effect on the yield of reactivation of ODH (Figure 4b). On the other hand, these additives have apparently no effect on the rate of reactivation (Figures 3c and 4a). In

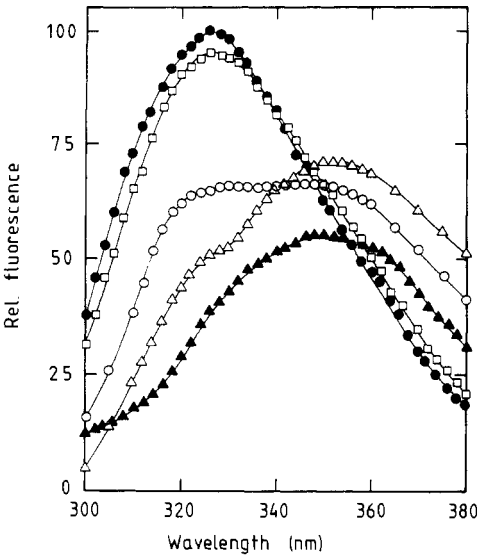


FIGURE 5: Fluorescence spectra of native ODH (●) in the absence of additives or in the presence of 25% PEG and of the denatured enzyme in 6 M Gdn·HCl (▲) in comparison to the protein renatured in the absence of additives (□) or in the presence of 10% PEG (1.75 cP) (○) or 25% PEG (4.0 cP) (△). Excitation wavelength was 280 nm; enzyme concentration was 4.5 μg/mL; temperature was 20 °C.

addition to its lower activity, the enzyme that has been reconstituted in the presence of PEG has altered fluorescence properties: the wavelength of the emission maximum shifts from 327 to 350 nm as the concentration of PEG increases (Figure 5). The additive has no effect on the fluorescence emission of native ODH. It seems that the presence of high molecular weight components can change the folding pathway and favor the incorrectly folded state rather than the native state as the final product.

Refolding of ODH As Measured by Recovery of Resistance to Proteolysis. Native ODH is not inactivated by a 2-min incubation with trypsin or thermolysin (see Methods). However, the same short treatment with proteases prevents ODH completely from reactivating when applied at the beginning of the refolding process (cf. Figure 1).

Protease pulse experiments can therefore be used to measure the formation of protease-resistant structures during the renaturation of ODH. The extent of reactivation is determined as a function of the time allowed for renaturation prior to the protease pulse. The same kinetics are obtained when either trypsin or thermolysin is used, corresponding to a first order reaction with a rate constant of 2×10^{-3} s^{-1} (Figure 6). When ODH is renatured under the present conditions of solvent and temperature, the protein becomes resistant to proteolysis significantly faster than it becomes enzymatically active. The rate constant for the masking of potential cleavage sites (2×10^{-3} s^{-1}) is about twice as large as that of the reactivation step (9×10^{-4} s^{-1}) (Figure 6).

The rate of the reaction that controls the formation of a protease-resistant conformation depends markedly on temperature, with an energy of activation of 82 kJ/mol. This value is smaller than that for the reactivation of ODH of about 112 kJ/mol (Zettlmeissl et al., 1984). Thus, an increase in temperature will increase the difference between the rates of regain of activity and of resistance to proteases.

The presence of up to 30% (v/v) glycerol in the renaturation buffer, which corresponds to a solvent viscosity of 3.0 cP, does not affect the rate at which ODH resumes its ability to resist proteolytic cleavage (Figure 7). Adding glycerol therefore increases the difference between the rate of recovery of resistance to proteases, which does not change (2×10^{-3} s^{-1})

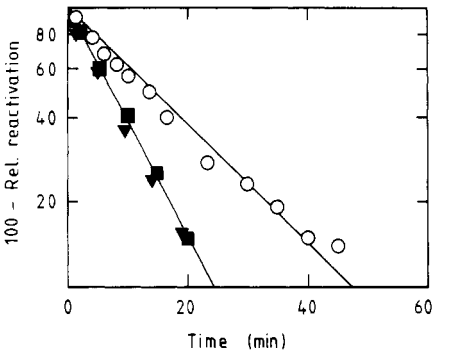


FIGURE 6: Time course of the recovery of resistance to proteolysis by 4 μg/mL trypsin (▼) or 10 μg/mL thermolysin (■) during refolding of ODH in comparison to the reactivation kinetics (○). Denaturation by 1-h incubation in 8 M urea, pH 3, at 20 °C. Refolding was performed at 20 °C, pH 7.6, in 0.1 M sodium phosphate, 1 mM dithioerythritol, 0.1 M residual urea, and, in the case of trypsin digestion, 1 mM EDTA. Protein concentration was 2.5 μg/mL. Proteolysis was stopped 2 min after addition of the protease by addition of either soybean trypsin inhibitor (trypsin) or EDTA (thermolysin).

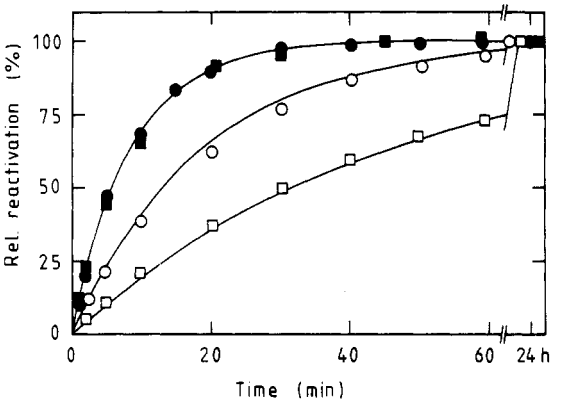


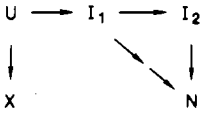
FIGURE 7: Influence of glycerol on the time course of the recovery of resistance to tryptic cleavage in comparison with the effect on the kinetics of reactivation. (Closed symbols) Resistance to proteolysis upon refolding in the absence of additives (●) and in the presence of 30% glycerol (3.0 cP) (■). (Open symbols) Reactivation in the absence of additives (○) and in the presence of 30% glycerol (3.0 cP) (□). Experimental conditions otherwise as described in Figure 6.

(Figure 7), and the rate of reactivation, which becomes slower (the rate constant in the presence of 30% glycerol is 3.7×10^{-4} s^{-1}, as compared to 9×10^{-4} s^{-1} in the absence of glycerol) (Figure 3b). The ratio of these rates increases from a value slightly above 2 in the absence of glycerol to a value close to 5 at 30% (v/v) glycerol. Even though the rates are of the same order of magnitude, the differences in the dependences on temperature and glycerol concentration clearly show that the formation of a protease-resistant structure and the reappearance of enzymatic activity are controlled by two different processes.

Discussion

Three different processes have been identified during the renaturation of ODH from the completely unfolded state (U) obtained after a long incubation in either 8 M urea at pH 3 or 6 M Gdn·HCl at pH 7.6. Refolding begins with the rapid formation of a structured intermediate (I_1). The polypeptide chain organizes itself into segments of secondary structure and compact regions, so that the resulting structure is stable enough to expel water and to shield the aromatic groups from the aqueous solvent. This first intermediate (I_1) possesses fluorescence and circular dichroism properties that are already identical with those of the native protein. The rapid U → I_1

step involves the entire change in the protein fluorescence. After I_1 has formed, the protein undergoes a slower reaction and acquires a conformation that is resistant to proteolysis (I_2). The same kinetics are obtained by use of two proteases with different specificities, trypsin and thermolysin, suggesting that a unique molecular process controls the sensitivity of several cleavage sites. The slowest process observed corresponds to the regain of the activity and to the reappearance of N, native ODH. If the two reactions, formation of a protease-resistant structure and reactivation, were consecutive, i.e., if N was produced only from I_2, one would expect reactivation to show a lag phase due to the time needed to form I_2 from I_1. The fact that no such lag phase is detected (Figures 2 and 7) indicates that only part of the ODH chains proceed through I_2, while the remaining molecules can form N directly from I_1:



(1)

In this mechanism, X stands for an incorrectly folded, inactive species (see below). The given mechanism is an extension of the previously proposed sequential mechanism (Zettlmeissl et al., 1984). According to this mechanism, the recovery of resistance to proteolysis measures the disappearance of I_1, and the return of activity measures the appearance of N. The difference between the rates of these two reactions indicates that only a minor fraction of the ODH chains takes the direct path from I_1 to N while a major fraction refolds by a sequential path via I_2. However, the "direct" $I_1 \rightarrow\rightarrow N$ pathway produces enough active molecules so that the lag predicted from the parallel sequential pathway ($I_1 \rightarrow I_2 \rightarrow N$) can no longer be detected. It could also be the case that the direct pathway involves an active intermediate.

The disappearance of I_1 via I_2 according to eq 1 could be related to the cis–trans isomerization of (some of) the X–Pro peptide bonds. Its rate of 2×10^{-3} s^{-1} at 20 °C (Figures 6 and 7) and its activation energy of 82 kJ/mol are comparable to those of proline isomerization (Brandts et al., 1975; Cook et al., 1979; Stellwagen, 1979; Garel, 1980). Also, the rate of the $I_1 \rightarrow I_2$ reaction in ODH does not change in the presence of glycerol (Figure 7), similar to the proline isomerization in ribonuclease which does not depend on solvent viscosity (Tsong & Baldwin, 1978; Schmid & Baldwin, 1979). ODH has 14 prolines, and it is likely that some of them are important for folding since most of the proteins studied up to now have a part of their folding that is rate-limited by the cis–trans isomerization of prolines (Kim & Baldwin, 1982; Kuwajima & Schmid, 1984). The disappearance of I_1 is measured by the recovery of the resistance to proteases. Two explanations can be proposed to relate the cis–trans isomerization of prolines to the disappearance of cleavage sites. The first possibility is that the segment(s) that is (are) susceptible to proteolysis is (are) no longer exposed in I_2 or N whereas it was (they were) accessible in U or I_1. In this case, I_2 would be more folded than I_1, so as to mask all the sites for both trypsin and thermolysin. The cis–trans isomerization of proline can also prevent the cleavage of a neighboring peptide bond when the protease accepts only one configuration of the proline as a substrate. Such isomeric specificity has been demonstrated for trypsin (Lin & Brandts, 1983) and could exist for other proteases as well.

Instead of folding into their native state, about 30% of the ODH chains form an incorrectly folded and inactive species (X), which is characterized by a maximum of fluorescence emission at 350–360 nm (Figure 5). It is more likely, as shown in eq 1, that X is formed from U, in which the tryptophans are exposed, rather than from I_1, in which the tryptophans are buried. The yield in the reactivation of ODH seems then governed by a competition between the correct folding reaction $U \rightarrow I_1$ and the incorrect side reaction $U \rightarrow X$. Although X does not form large aggregates (Zettlmeissl et al., 1984), it cannot convert into the native state N at any measurable rate.

The presence of high molecular weight additives, PEG and PVP, decreases the yield of renaturation without affecting its rate (Figures 3c and 4). They favor the formation of the incorrectly folded state (X), as judged from the fluorescence spectrum of the reconstituted protein (Figure 5). These additives seem to interfere with renaturation at an early stage preceding the formation of I_1. It is known that the phase separation between a macromolecule and the solvent can be largely modified by the presence of another polymeric component (Flory, 1953). The effect of PEG on protein stability has been discussed in detail by Lee and Lee (1981) and Knoll and Hermans (1981). Folding of the ODH chain into I_1 implies the expulsion of water from compact regions and the formation of a nonsoluble phase. The state X represents a more soluble phase (much less shielding of the tryptophans from solvent is observed), which is more stable in the presence of PEG or PVP. High molecular weight additives do not seem to affect the reactivation of ODH through their effect on viscosity but rather through their polymeric nature, which perturbs the first folding steps.

The slowest reaction measured by the recovery of the enzymatic activity corresponds to the formation of the native protein. The reactivation of ODH is slower in the presence of low molecular weight additives, glucose or glycerol (Figures 3a,b and 4a), but its yield remains the same (Figure 4b). Glycerol and, to a lesser extent, glucose not only increase the viscosity of the solvent but also stabilize the structure of proteins (Gekko & Timasheff, 1981a,b; Arakawa & Timasheff, 1982). In the presence of glucose and glycerol, proteins are preferentially hydrated, i.e., addition of these components to an aqueous solution of a protein results in an unfavorable free energy change (Arakawa & Timasheff, 1982). The magnitude of this change in free energy increases with the area of contact between the protein and the solvent. In the presence of these additives as well as that of sucrose (Lee et al., 1975) and some salts (Aune & Timasheff, 1970; Timasheff et al., 1976; Arakawa & Timasheff, 1982), the protein will minimize the unfavorable energy change by decreasing the part of its surface that is accessible to the solvent. The same decrease in the surface-to-volume ratio explains the ability of all these agents to stabilize protein structure, at the level of both intra- and interchain interactions. However, the influence of glycerol on the rate of reactivation of ODH does not result primarily from its stabilizing properties: this rate is not modified by a stabilizing agent, $(NH_4)_2SO_4$, at a concentration of 0.5 M, or by the coenzyme NADH, which also stabilizes the native structure (Zettlmeissl et al., 1984). It seems then as if the major effect of glycerol and glucose on the rate of ODH reactivation is due to the increase in solvent viscosity. Figure 4a shows that the friction exerted by the solvent, measured as the reciprocal of the reactivation rate, increases linearly with its viscosity. Glucose seems to be somewhat more effective than glycerol, with a slope of 0.9 for the former as compared to 0.6 for the latter. For the same solvent viscosity, the influence of glycerol could be due to a major viscosity effect (similar to that seen with glucose) partly counterbalanced by a minor stabilizing effect.

Glycerol does not change the rate of formation of the protease-resistant structure (Figure 7) and decreases that of reactivation (Figures 3a, 4a, and 7). It seems then that solvent viscosity affects a late stage of ODH folding, probably the $I_2 \rightarrow N$ reaction which produces most of the native state. In the case of ODH, as well as of aspartokinase–homoserine dehydrogenase (Vaucheret et al., 1987), the low molecular weight additives like glucose, glycerol, or sucrose influence the rate of protein renaturation at the last step through their viscosity properties. The formation of N, which occurs mostly by the $I_2 \rightarrow N$ reaction, is dependent on solvent viscosity and thus probably involves the relative movement of two parts of the ODH molecule. This reaction takes place in the already largely folded intermediate I_2 and could correspond to the displacement through solvent of a compact region rather than to the local adjustment of a few residues.

The structure of ODH is likely to be similar to that of other NAD-dependent dehydrogenases and thus to be composed of two domains corresponding to two different segments along the polypeptide chain. In the native state, one domain is involved in binding the coenzyme and the other in binding the substrate(s). The active site is constituted by the interface between the NAD- and the substrate-binding domains (Grau, 1982). In the few cases of multidomain proteins that have been studied, it has been found that each domain behaves as an independent folding unit (Crisanti & Matthews, 1981; Dautry-Varsat & Garel, 1981; Zetina & Goldberg, 1982), and this holds probably also for ODH. The renaturation of ODH would begin by the independent folding of its (two) domains to produce a folded and inactive intermediate. The reaction that leads to the reappearance of the enzymatic activity implies the formation of a functional active site, i.e., the correct mutual adjustment of the NAD- and substrate-binding domains. It is therefore plausible to attribute the $I_2 \rightarrow N$ reaction to the pairing of the two domains that constitute the native ODH. This reaction takes place in a folded state and produces active ODH, and its rate is viscosity-dependent as expected if these two domains move through the solvent to find each other. For most of the ODH molecules, reactivation is rate-limited by the $I_2 \rightarrow N$ step, and it is rather surprising that this domain-pairing step is the slowest of the overall folding process. A similar interpretation has been proposed for other large polypeptide chains (Carrey & Pain, 1978; Creighton & Pain, 1980; Beasty et al., 1986; Vaucheret et al., 1987), which suggests that such a slow domain-pairing reaction could be a general feature of the renaturation of large proteins. The process that limits the rate of protein folding would depend on the size of the polypeptide. Proline cis–trans isomerization, which seems to be limiting for small proteins, probably also takes place in large proteins. However, it is no longer critical for the rate of folding, which would be controlled by the pairing of already folded domains.

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