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Review

Folding and Association of Proteins*

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Abstract. The acquisition of the native three-dimensional structure of proteins consists of sequential folding reactions with well-populated and well-defined structural intermediates. For small proteins successive stages in the folding have been resolved kinetically; these suggest that H-bonded elements of secondary structure are formed first, followed by folding steps to generate the complete tertiary structure.

The rate determining step in the folding of a number of small proteins has been shown to be proline *cis* $\rightleftharpoons$ *trans* isomerization. As indicated by experiments using fast kinetics the overall folding mechanism, even in a small single-domain molecule like ribonuclease, involves more than one intermediate.

Large protein molecules contain domains which may fold independently. For multi-domain proteins, the pathway of folding therefore involves "folding by parts", followed by merging of folded domains.

In the case of assembly systems (e.g., oligomeric or multimeric enzymes) folding and association have to be subtly interconnected with respect to the time scale, since the correct assembly of subunits requires their proper folding. In this sense the initial function of oligomeric proteins is their own self-assembly. The corresponding mechanism underlying the spontaneous formation of the native quaternary structure of oligomeric proteins must be the consecutive folding and association of the constituent polypeptide chains.

Equilibrium and kinetic studies have been concerned with a number of dimeric, tetrameric and multimeric enzymes, using enzymatic activity to measure structure formation: alcohol dehydrogenase, aldolase, glyceraldehyde-3-phosphate dehydrogenase, lactic dehydrogenase, malic dehydrogenase, pyruvate dehydrogenase, triose phosphate isomerase, tryptophan synthase.

* Dedicated to Professor Ernst M. Helmreich on the occasion of his sixtieth birthday

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These experiments make use of the reversibility of protein denaturation, focusing on refolding and reassociation rather than folding and association, because there is no direct approach to structural investigations of the nascent polypeptide chain in vivo.

Optimum conditions of reconstitution yield up to 100% reactivation. After separation of "irreversibly denatured protein", reconstituted and native protein turn out to be indistinguishable. The major side reaction leading to "wrong aggregation" is due to competition between folding and association.

Due to the high specificity of the association reaction "chimeric" species are not observed, and multimeric systems containing different component enzymes show specific assembly.

The kinetics of reconstitution generally obey an irreversible sequential first- order/second-order mechanism involving inactive monomers; only in the case of aldolase is subunit activity suggested. For a number of oligomeric enzymes renaturation from various denaturants, in the absence or presence of coenzyme is characterized by identical kinetics. For glyceraldehyde-3-phosphate dehydrogenase, however, free NAD as well as a covalently bound NAD-analog are found to enhance the reconstitution.

In the case of assembly structures exceeding the dimer, the observed consecutive folding/association mechanism does not allow us to decide whether the observed second order processes belong to the formation of the dimer or tetramer. Chemical cross-linking and hybridization techniques allow the equilibrium state and the assembly kinetics of oligomeric systems to be analyzed quantitatively. Using this method, e.g., for lactic dehydrogenase, it is obvious that dissociation leads to the homogeneous monomer, while tetramer formation is found to parallel reactivation.

In general, equilibrium and kinetic experiments prove that full enzymatic activity requires association.

In the case of multisubunit enzymes (multienzyme complexes) heterologous interactions of the component enzymes seem to be involved in the rate determining (first order) "reshuffling" processes which generate catalytic activity in the overall enzymatic reaction.

Key words: Association – Folding – Oligomeric enzymes – Proteins – Reconstitution

Introduction

The molecular mechanism of protein biosynthesis has been elucidated in recent years. The organelles and factors involved are known, the sequence of events established, and the observed fidelity may be explained by synergistic conformational effects at the tRNA and synthetase level, as well as kinetic proof reading processes.

A problem that remains to be solved is the post-translational (or syn-translational) acquisition of the functional state of the nascent polypeptide

chain. The process seems to be based solely on the amino acid sequence which has been generally shown to determine the three-dimensional structure. We call the transition from the one-dimensional to the three-dimensional structure "folding" (Jaenicke 1980; Thomas and Schechter 1980; Richardson 1981).

Considering the vast amount of experimental data obtained for a number of small single-chain proteins the stage is set for determining the pathway of folding and for characterizing the structure of folding intermediates (Baldwin 1975; Baldwin and Creighton 1980).

In going from single-chain proteins to dimers, tetramers, or (more general) oligomers, the association of polypeptide chains has to be considered in addition to folding. Both processes must be properly coordinated because the formation of the native quaternary structure requires the surfaces of the structured monomers to be preformed in the correct way such that specific recognition is achieved (Jaenicke 1978).

As indicated by experiments involving reversible denaturation-renaturation of a wide variety of proteins, folding and association occur spontaneously without the need for any additional information beyond that contained in the specific amino acid sequence on one hand, and the aqueous or non polar environment (for cytoplasmic or membrane proteins, respectively) on the other. This finding has been considered to prove that the protein in its native three-dimensional structure occupies the global minimum of potential energy rather than a local one. Neither the given argument nor structural predictions based on semi-empirical potential functions are suited to decide whether the native structure is in fact at the global minimum (Anfinsen and Scheraga 1975; Wetlaufer and Ristow 1973).

The kinetics of folding seem to contradict a purely thermodynamic concept of folding: comparing the time scale of in vivo folding[1] to the time required to probe all conformations in search of the global energy minimum, a difference of about 60 orders of magnitude is predicted (Levinthal 1968). To explain this discrepancy kinetic and configurational constraints must be effective ("nucleation", excluded volume effects, "folding by parts" etc.). The importance of these effects is illustrated by the unsuccessful attempts to calculate the three-dimensional structure of proteins ab initio on the basis of energy minimization, using bond angles and lengths in the framework of Ramachandran's concept of accessible configurational space (Anfinsen and Scheraga 1975; Schulz and Schirmer 1979; Levitt 1980).

Structure Formation in Vivo and in Vitro

The experiments underlying the following discussion make use of the reversibility of protein denaturation focusing on *re*folding and *re*association rather than folding and association. The reason is that so far no direct approach

1 For the total enzyme pattern, e.g., of Escherichia coli the upper limit for the time required for folding and association is the generation time of the bacterium (~ 20 min). The folding of an average protein of $M_r \sim 100{,}000$ takes about 2 min

Table 1. Experimental approaches to the folding and association of proteins

Equilibrium measurements	
State of association	Electron microscopy, Ultracentrifugation, (quasielastic) light scattering, gel permeation chromatography, SDS polyacrylamide gel-electrophoresis (with and without crosslinking)
Conformation	Spectroscopy (absorption, fluorescence, optical rotatory dispersion, circular dichroism), hydrogen-deuterium (tritium) exchange, stability towards denaturation or proteolysis, binding of ligands (coenzymes, substrates etc,), chemical modification ("group specific" labels)
Function (Activity)	Enzymatic assays, ligand binding (affinity chromatography)
Kinetic measurements:	*Mix, stopped flow, double jump, relaxation techniques*
Assembly (association)	Turbidity, (quasielastic) light scattering, chemical crosslinking, hybridization
Folding	Spectroscopy (absorption, fluorescence, circular dichroism), hydrogen-deuterium exchange, limited proteolysis (fragment analysis)
Function (activity)	Enzymatic assays, ligand binding

is available to study the acquisition of the native structure of the nascent polypeptide chain either in vivo or in vitro. Experimental approaches used to investigate the folding and association of oligomeric systems are summarized in Table 1.

Evidently the in vitro reconstitution may be different from the vectorial in vivo folding process, because refolding starts from the denatured complete chain while folding of the nascent protein may occur as a syn-translational event (Fig. 1). The question of whether refolding indeed reflects the in vivo process cannot be answered unambiguously. However, two criteria suggest that the two reactions follow similar pathways: (*i*) under certain experimental conditions the kinetics of reconstitution are in the same time range as the folding of nascent polypeptide chains in vivo; (*ii*) the final product of reconstitution after denaturation – renaturation is found to be indistinguishable from the initial native state (Jaenicke and Rudolph 1980).

The latter finding has been documented by a variety of physicochemical and enzymological methods. For some small single-chain proteins it holds without restriction and the transition $N \rightleftharpoons D$ between the native and denatured states may be considered a completely reversible reaction adequately described by a two-state model ($\Delta H_{\text{calorimetric}}/\Delta H_{\text{van't Hoff}} \sim 1$, cf. Pfeil 1981). For most large (oligomeric) proteins the $N \rightleftharpoons D$ transition is characterized by a range of metastability giving rise to "hysteresis" and "wrong aggregation" (Fig. 2).

In general, deactivation, unfolding and dissociation run parallel. The same holds for reactivation, refolding and reassociation. Therefore, studying the equilibrium properties in the oligomer transition range normally does not answer the question of whether or not isolated monomers of oligomeric enzymes possess catalytic function, or maintain at least part of the structural properties characteristic for the native enzyme. In this connection kinetic reconstitution

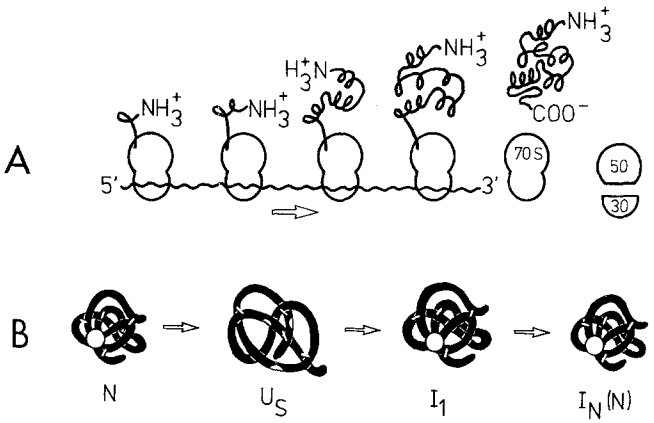


Fig. 1A and B. Folding in vivo and in vitro. **A** Ribosomes moving along the m-RNA in 5′ → 3′ direction release the growing polypeptide chain. Folding may occur either as a syn-translational or post-translational event. **B** Unfolding and refolding of a single-chain, one domain protein (e.g. ribonuclease) demonstrating the non-vectorial character of in vitro folding. For symbols see Eq. (1) and Eq. (2)

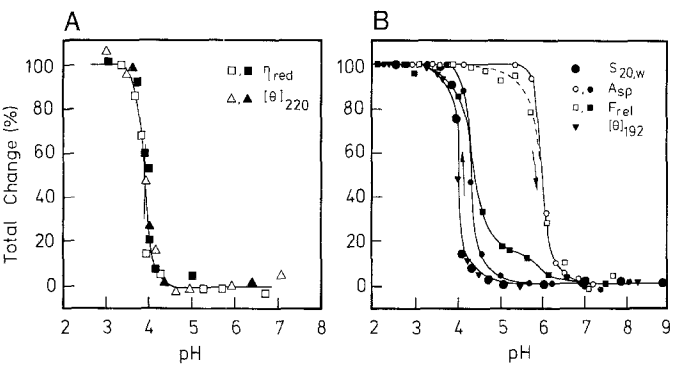


Fig. 2A and B. Denaturation-renaturation of single-chain and multi-chain proteins. **A** *Staphylococcal nuclease:* change in reduced viscosity and molar ellipticity at 220 nm as a percentage of the total change. Open symbols refer to denaturation (addition of acid), closed symbols to renaturation (addition of base). **B** *Lactic dehydrogenase* from pig skeletal muscle in 0.1 M phosphate (+ 1 mM EDTA + 0.1 mM dithioerythritol): change in sedimentation coefficient (c = 0.36 mg/ml), enzymatic activity (c = 9 μg/ml), rel. fluorescence at λ = 340 nm (λ_{exc} = 275 nm, c = 9 μg/ml), ellipticity at λ = 192 nm (c = 0.36 mg/ml)

studies have been shown to provide a powerful tool to investigate the correlation of folding and association.

There is a wide variety of parameters affecting the unfolding and reconstitution of protein structure: pH, temperature, hydrostatic pressure, "structure breaking" or "structure making" solvent components (like guanidine · HCl, or Na_2SO_4, among others). In the present context three aspects of this variety are of importance: (*i*) For the experimentalist the choice of proper conditions provides a means to adjust the folding-unfolding reaction with respect

to its yield and kinetics. To give an example, reconstitution and "wrong aggregation" compete with each other upon reactivation of oligomeric enzymes depending on protein concentration (Jaenicke 1974; Zettlmeißl et al. 1979a). (*ii*) The various denaturants cause different levels of structural perturbation, ranging from "very native conditions" to "complete denaturation". As a consequence, renaturation may refer to a whole spectrum of processes the kinetics of which may be applied to analyze the multiple pathways and elementary processes of folding. (*iii*) The physiological conditions do not necessarily coincide with the conditions of maximum stability of a given protein. This observation may be merely a consequence of the fact that proteins are multifunctional systems, and that it is impossible to optimize simultaneously functionality *and* stability in a single device, especially because structural flexibility is essential for both enzyme function and protein turnover (Wetlaufer 1980; Jaenicke 1981).

Considering the various modes of denaturation and refolding it is not surprising that under certain conditions well-populated intermediate states of folding and association do exist whereas in others apparent two-state behaviour is observed (Kim and Baldwin 1982). In monomeric systems the intermediates may be used to analyze the folding pathway; in oligomeric enzymes partially structured intermediates allow the correlation of catalytic function with single events in the multi-step assembly process.

Folding

In discussing the "code" for the formation of the three-dimensional structure of protein assemblies we first consider the mechanism of folding of a single-chain, one domain protein like ribonuclease (RNase, Fig. 3). For reviews see: Baldwin 1975; Baldwin and Creighton 1980; Kim and Baldwin 1982.

As taken from the complete reversibility of denaturation, a molecule like this folds to a well-defined (thermodynamically stable) structure which turns out to be identical with the native state (*N*), in spite of the great number of possible configurations of a polypeptide chain of 124 amino acids. The process is found to be exceedingly fast, indicating some kind of kinetic control.

As indicated, the "code" for the chain folding must be intimately connected with the primary sequence of the protein. Considering the functional convergence in protein families like NAD dependent dehydrogenases or heme proteins, specific "folds" may show only little homology in sequence. Thus the folding code, as well as the folding pathway are expected to be complex. However, they may very well be general in the sense that classes of "folds" correspond to certain folding pathways. Experimental attempts to elucidate both are hampered by the high rate of the elementary processes of folding[2], and by the cooperative nature of the folding-unfolding transition. Therefore, it is difficult to distinguish between the numerous theoretical kinetic and structural models available (for models see Kim and Baldwin 1982).

2 For example α-helix formation in poly-amino acids and poly-peptides occurs in the time range of μs (Hammes and Roberts 1969, Barksdale and Stuehr 1972; Gruenewald et al. 1979)

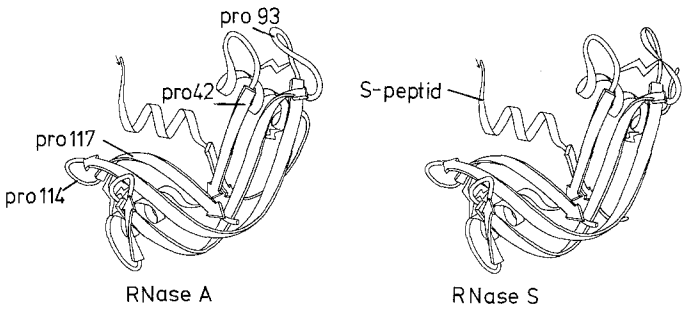


Fig. 3. Schematic drawing of the backbone of *ribonuclease A* and *ribonuclease S*, demonstrating the positions of proline residues and the site of subtilisine cleavage (adapted from Richardson 1981)

A plausible model of protein folding which has its analog in multistep transitions upon protein denaturation (Tanford 1968, 1970) is "sequential folding", i.e., folding occurring in a unique and (under definite experimental conditions) well-defined sequence of steps. Specific intermediates have been shown to be well-populated in the folding of a number of single-chain proteins.

Single-Chain, One-Domain Proteins

Most experimental work has been done with RNase A (Baldwin 1980). The most fascinating feature of the folding of this enzyme is the finding that the folding reaction clearly indicates at least two kinetically separable unfolded states (U_F, U_S), while the thermodynamics of the $N \rightleftharpoons D$ transition may be quantitatively described by a two-state process. The corresponding minimal kinetic scheme must be

$$N \rightleftharpoons U_F \rightleftharpoons U_S \,. \tag{1}$$

Analyzing the spectroscopically distinct species the following picture emerges:
(*i*) The two unfolded species U_S and U_F give rise to two separate refolding reactions which both yield native enzyme:

$$U_S \rightarrow N \quad (80\%)\ \tau_{1/2} = 12\ \text{s}\,,$$
$$U_F \rightarrow N \quad (20\%)\ \tau_{1/2} = 30\ \text{ms}\,.$$

(*ii*) The activation energy for the $U_F \rightleftharpoons U_S$ equilibrium is ~ 85 kJ/Mol.
(*iii*) The relative concentrations of the fast and slow folding species are not affected by high temperature or strong denaturants. Therefore U_S and U_F cannot be partly folded: they must differ entropically rather than energetically, in accordance with the above mentioned two-state model.

A possible mechanism explaining the existence of multiple unfolded species is the proline isomerization model proposed by Brandts et al. (1975). According to this model in the native state each proline peptide bond shows a defined configuration (either *cis* or *trans*). After rapid unfolding ($N \rightarrow U_F$) the prolines

Table 2. Comparison of the $U_F \rightleftharpoons U_S$ transition of RNase *A* with proline *cis* $\rightleftharpoons$ *trans* isomerization

	RNase *A* $U_F \rightleftharpoons U_S$[a]	Proline *cis* $\rightleftharpoons$ *trans*[b]
Equilibrium		
K_{eq}	0.25	0.1–1.0
$K_{eq} = K_{eq}$ (denaturant)[c]	no	no
ΔH	0 kJ/mol	0–4 kJ/mol
Kinetics		
$\tau_{1/2}$ (25° C)	40 s	10–100 s
$\tau = \tau$ (denaturant)[c]	no	no
$\Delta H^{\neq}$	85 kJ/mol	84 ± 12 kJ/mol
Acid catalysis	in $\geqq 5\ M\ HClO_4$	in strong acids

[a] Schmid and Baldwin 1978
[b] Brandts et al. (1975)
[c] Dependence on pH or guanidine · HCl

isomerize to a *cis* $\rightleftharpoons$ *trans* equilibrium mixture (in the $U_F \rightleftharpoons U_S$ step) with a typical ratio of 80% *trans* and 20% *cis* configuration. The unfolded species U_F contains all essential prolines in the native configuration, and therefore refolds rapidly; U_S contains at least one non-native proline isomer the isomerization of which slows down the rate of refolding.

Recent experiments indicate that under folding conditions of marginal stability the $U_S \rightarrow N$ folding pathway is adequately described in terms of a two-state process which is rate-limited by proline isomerization (Table 2). Under "strongly native" folding conditions, however, two folding intermediates have been detected and characterized in structural terms. The corresponding refolding mechanism

$$U_S \rightarrow I_1 \rightarrow I_N \rightarrow N \qquad (2)$$

contains an intermediate I_1 with an open conformation and hydrogen-bonded secondary structure formed rapidly at an early stage of folding (Schmid and Baldwin 1979). I_N is a native-like intermediate with a hydrophobic interior and a nucleotide binding site as well as catalytic activity similar to the native enzyme. It differs from N in one incorrect proline isomer and shows only slight spectral alterations.

If we try to transfer the foregoing results to a hypothetical folding mechanism of the nascent polypeptide chain, we may postulate that the all-*trans* polypeptide chain formed on the ribosome in a first rapid reaction forms α-helices and β-structures. In a second, slow step, proline residues (preferably in β-bends) undergo *trans-cis* isomerization. If this reaction does participate in protein folding in vivo, the respective "hinge-residues" may serve the purpose of keeping the nascent molecule sufficiently flexible to allow the final energy minimization.

Disulfide bridges, the cross-linking of which is another late event in structure formation, seem to have merely a stabilizing effect. As indicated by reduction-reoxidation experiments their formation follows a well-defined sequence (Fig. 4).

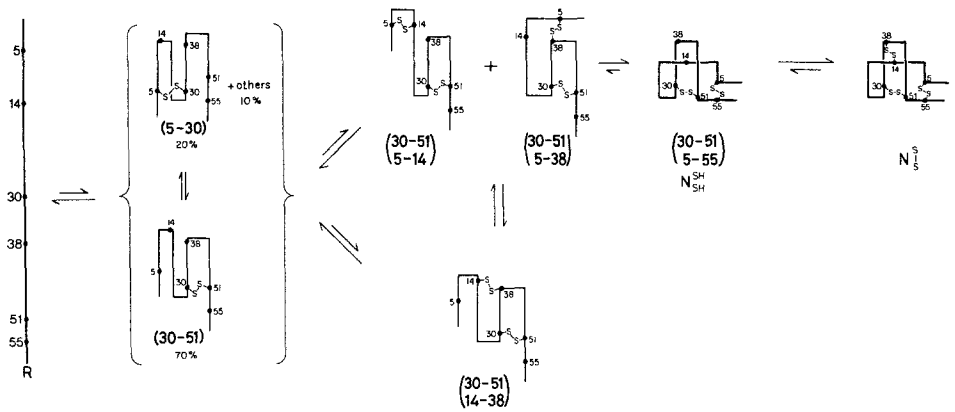
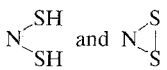


Fig. 4. Schematic diagram of the folding pathway of bovine pancreas trypsin inhibitor. The solid line on the left represents the polypeptide chain, with the positions of the six cysteine residues indicated. The configurations of species



approximate the native conformation. Cysteine residues involved in intermediary disulfide bonds are indicated below the schematic of each of the folding intermediates. Single-disulfide intermediates in { } are in rapid equilibrium; only the most prominent species are given (cf. Creighton 1980)

Folding by Parts

In going from small single-chain proteins to oligomeric systems, two aspects need consideration:

(*i*) In general the subunit molecular weights of multimeric systems exceed the molecular weight of the small polypeptide chains discussed so far. An increase in chain length and in the number of proline residues both tend to increase the half-time of folding (Lin and Brandts 1978). Correspondingly, the folding rate of oligomeric systems with an average molecular weight $\mathbf{M}_r \sim 40{,}000$ is about two orders of magnitude slower than the rate of RNase folding.

(*ii*) Most of the previously mentioned larger molecules are in fact composed of "domains", i.e., compact local regions of structure, or folding units, characterized by continuous sequences of polypeptide chain of the order of about 10,000–15,000 molecular weight. They may originate from gene duplication, joining of exons, or deletion of stop codons. In cases where they are clearly separated from vicinal structures each domain may fold independently, with the joining of the folded units as a rate-limiting step in the formation of activity (Wetlaufer 1981; Goldberg and Zetina 1980). Obviously, "folding units" preserved as domains in the native protein may be considered obligatory kinetic intermediates on the folding pathway. Their selective advantage is evident; namely an accelerating effect on the overall folding process, and the possible reduction of proteolysis and "wrong aggregation" (see below). To give an example, monomeric staphylococcal penicillinase ($\mathbf{M}_r = 29{,}000$) contains three "domains" which fold independently (Adams et al. 1980; Creighton and Pain

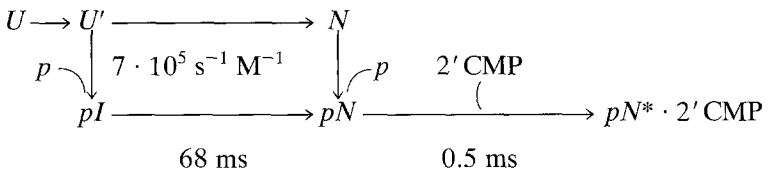
1980). In certain enzymes such domains may have different enzymatic functions as in the case of the tetrameric aspartokinase-homoserine dehydrogenase. This enzyme represents a bifunctional oligomer and illustrates the combination of local domain structure and global allosteric function (Dautry-Varsat and Garel 1981, cf. Wetlaufer 1981).

A characteristic feature of proteins containing independent folding units is the occurrence of equilibrium intermediates indicated, e.g., by biphasic unfolding transitions detected spectroscopically or calorimetrically (Pfeil 1981).

Fragment Studies

The simplest possible model for intermediate states in the process of folding and association is the reconstitution of protein fragments. If we assume that protein folding consists of the consecutive formation of microdomains → subdomains → domains, which finally coalesce into the native three-dimensional structure, then appropriately chosen fragments should be good candidates to investigate the mutual effect of subunits on their folding during quaternary structure formation. It has been frequently demonstrated that protein fragments may fold independently (Wetlaufer 1981). However, fragments of the previously mentioned small single-chain proteins, like the S-peptide (residues 1–20) that complements S-protein (residues 21–124) in the case of RNase (Fig. 3, cf. Richards and Wyckoff 1971) are found to be unfolded under standard refolding conditions unless they are recombined to form the quasi-native RNase complex (Labhardt and Baldwin 1979, Labhardt 1980, Bierzynski et al. 1982).

Kinetic studies have shown that the unfolded S-peptide (p) binds to an early folding intermediate (U') containing some β-structure. Subsequent S-peptide α-helix formation parallels the regain of substrate binding capacity, according to the kinetic scheme



(3)

with U, U' = unfolded states, I = intermediate, N, N^* = native states, and p = S-peptide. Since the product of recombination, pN^*, shows the characteristics of the native enzyme, this model reaction, for the first time, illustrates the correlation of folding, association and enzymatic activity that is typical for oligomeric enzymes.

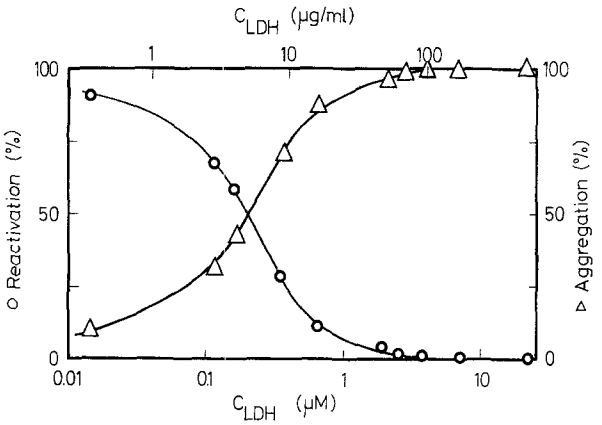


Fig. 5. Reconstitution of lactic dehydrogenase from pig skeletal muscle: Effect of enzyme concentration on the relative extent of reactivation and non-covalent aggregation after denaturation in 0.1 M H_3PO_4 pH 2.5 (+ 1 mM EDTA + 1 mM dithioerythritol, 20° C, ca. 5 min). Reactivation in 0.1 M phosphate buffer pH 7.0 (+ 1 mM EDTA + 1 mM dithioerythritol, 20° C, up to 192 h). At $c < 10$ nM reactivation yields > 90% (Zettlmeißl et al. 1979a)

Association

Reconstitution vs Aggregation

The foregoing model reaction differs from the general situation during folding and association of oligomeric proteins in two ways. Firstly, quaternary structure formation refers to polypeptide chains of comparable size which on principle exceed the subdomain size, so that solvent interactions enforce a certain intrinsic backbone structure. Secondly, in contrast to small single-chain systems and subdomain fragments, subunits of oligomeric proteins show a significant competition of intra- and intermolecular interactions giving rise to incomplete reconstitution due to "wrong aggregation" (Teipel and Koshland 1971; Jaenicke 1974; Zettlmeißl et al. 1979a).

While the S-peptide of RNase forms its α-helical structure only imperfectly unless the *pN** complex is formed (Labhardt 1980; Bierzynski et al. 1982), oligomeric systems are found to depend on the formation of "structured monomers" as a prerequisite of correct association, i.e., proper subunit folding providing complementary surface areas has to precede subunit association.

The "tuning" of subunit folding and assembly becomes obvious upon changing the relative rates of the two processes by varying protein concentration. As shown in Fig. 5 reconstitution and formation of "wrong aggregates" in the case of lactic dehydrogenase complement each other suggesting kinetic competition of the two processes according to

$$\begin{array}{l} D \xrightarrow{\text{first order}} N \\ \downarrow \text{ higher than first order} \\ A \end{array} \qquad (4)$$

with D, N, and A as denatured, reconstituted (= native), and aggregated states of the enzyme.

The aggregates are composed of individual monomeric chains with partially restored backbone structure (Zettlmeißl et al. 1979a). The kinetics of their formation are determined by a process with a reaction order greater than two competing with a first-order folding reaction in the pathway of reactivation. Both are kinetically controlled; the ratio of the two fractions depends solely on the ratio of the rates of their formation, and not on the relative conformational energies of the native enzyme and its aggregates. As in the case of high protein concentration, long incubation at low pH leads to a decreased yield of reactivation due to a deceleration of correct reconstitution which shifts the competitive reactions towards aggregation. Far-UV circular dichroism measurements suggest slow rearrangements within the (partially) unfolded monomers to be responsible for additional slow reshuffling reactions during reconstitution (Zettlmeißl et al. 1981). The corresponding kinetic scheme would be

$$\begin{array}{ccccc} D^* & \rightleftharpoons & D & \rightarrow & N \\ \downarrow & & \downarrow & & \\ A & & A & & \end{array} \qquad (5)$$

Reconstitution after "complete unfolding" in guanidine · HCl is characterized by a similar scheme. Proline *cis* ⇌ *trans* isomerization in the denatured state is not involved in the above competitive mechanism (Zettlmeißl et al. 1982).

The aggregates are stabilized by non-covalent interactions and perfectly stable under non-denaturing conditions. As long as cystine cross bridges can be excluded, their degradation is provided by strong denaturants (e.g., 6 M guanidine · HCl) which free the randomized individual chains to regain their native conformation and biological function after readjusting to optimum reconstitution conditions (Rudolph et al. 1979).

Folding and Association

In vitro reconstitution depends on the state of denaturation and the relative rates of folding and association. Due to the fact that dissociation, deactivation and denaturation of oligomeric enzymes, and the respective reverse reactions are coupled processes (cf. Fig. 2) association can not be totally separated from folding. Therefore, investigations of the equilibrium properties in the oligomer-monomer transition range (even if they were not affected by "hysteresis") are unable to answer the question of whether or not isolated monomers of oligomeric proteins possess biological function, or maintain at least part of the specific structural properties essential for the native state. The only way to obtain insight into the correlation between folding and association is by a kinetic analysis of the reconstitution reaction under a variety of different folding conditions and starting from different states of unfolding (Jaenicke 1974, 1978, 1980).

There have been successful attempts to achieve dissociation of oligomeric enzymes without drastic perturbation of the native backbone structure of the constituent subunits. In connection with the previously mentioned kinetic approach this is clearly indicated by the fact that rate-determining first-order folding reactions which are often found to precede association become insignificant.

Three types of experiments may serve as examples, at the same time illustrating various modes of dissociation-reassociation.

Structure Making Additives. Applying protective additives, like 1M Na_2SO_4, during acid dissociation of lactic dehydrogenase generates "structured monomers" providing an increased yield of reconstitution due to the elimination of the $D \rightarrow D^*$ transconformation in the denatured state [cf. Eq. (5); Hermann et al. 1981].

Cold Inactivation. Low temperature has been frequently found to cause inactivation of enzymes (Jaenicke 1981). The "cold inactivation" of yeast glyceraldehyde-3-phosphate dehydrogenase in the presence of ATP is accompanied by dissociation to monomers which are still capable of binding NAD (Bartholmes and Jaenicke 1978). This clearly indicates the overall structure of the enzyme with its complex nicotinamide and adenine binding domains to be preserved upon subunit dissociation. The dissociation kinetics show the typical low rate characteristic for structural transitions close to the equilibrium (Tanford 1968; 1970), while the kinetics of reconstitution depend on enzyme concentration, as expected for a rate determining association step. The overall process is quantitatively described by a sequential first order – second order reaction (see below, Fig. 6).

High Pressure Dissociation. A similar picture emerges if high hydrostatic pressure is applied to achieve subunit dissociation (Jaenicke 1981). In the case of lactic dehydrogenase, pressures in the range of $\leqq$ 1,000 bar lead to complete, reversible dissociation to inactive monomers (Schade et al. 1980; Müller et al. 1981) (Fig. 7). Monitoring the residual activity and the intrinsic fluorescence, deactivation is found to parallel structural transitions. Applying 8-anilino naphthalene sulfonic acid as an extrinsic fluorescent label, no significant increase in fluorescence is observed over the whole transition range at up to 1,500 bar, indicating that no significant unmasking of hydrophobic surfaces occurs upon subunit dissociation. On the other hand, X-ray analysis of the tetramer clearly shows the major contributions of hydrophobic residues to the intersubunit contact areas. We must therefore conclude that hydrophobic side-chains are buried in the process of high pressure dissociation.

As in the previous cases, reactivation and reassociation obey second-order kinetics, suggesting that the monomers possess no catalytic function. However, it cannot be decided without further experimentation whether association to the dimeric intermediate or to the native tetramer is rate-determining in the process of reactivation.

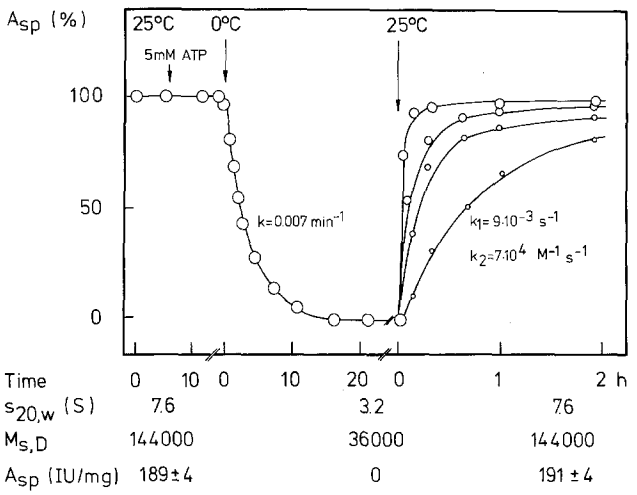


Fig. 6. Cold inactivation of yeast glyceraldehyde-3-phosphate dehydrogenase in the presence of 5 mM ATP. 0.1 M Tris buffer pH 8.5 plus 0.1 M 2-mercaptoethanol. *Dissociation* at 0° C, c_{GAPDH} = 1 mg/ml, with k = first-order rate constant. The monomer is capable of binding 1.1 ± 0.1 mol of NAD^+ (K_d = 98 ± 7 μM). *Reconstitution* at 25° C, c_{GAPDH} (in nM, based on $\mathbf{M}_r$ = 144,000): 70 (○), 27 (○), 13 (○), 6 (○). Solid lines calculated according to a consecutive folding-association mechanism $2A \overset{k_1}{\rightarrow} 2B \overset{k_2}{\rightarrow} C$ with k_1 and k_2 as first- and second-order rate constants (data from Bartholmes and Jaenicke 1978)

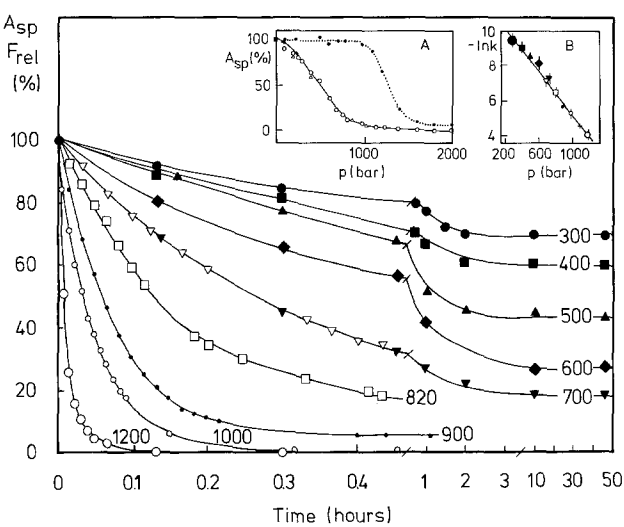


Fig. 7. High pressure deactivation and denaturation of lactic dehydrogenase from pig heart (apoenzyme). Tris buffer pH 7.6 (I = 0.16 M + 1 mM EDTA + 10 mM dithioeryhtritol, 20° C, c_{LDH} = 25 μg/ml). *Filled symbols:* kinetics of deactivation; *open symbols:* kinetics of the decrease in fluorescence emission at 345 nm (λ_{exc} = 285 nm). Isobars at pressures given in bar. *Insert A.* Pressure dependence of deactivation and reactivation. (○) Deactivation for ≧ 15 h at given pressure. (●) Reactivation yield of (○) after 24 h reconstitution at 1 bar. Deactivation for 20 min at 1,200 bar and reactivation at pressures < 1,200 bar yield the same profile. *Insert B.* Determination of the activation volume of deactivation and denaturation, calculated from the kinetics of deactivation and denaturation (k = first-order rate constant). Symbols as used for the isobars. $\Delta V^{\neq} = -140 \pm 10\ cm^3 \cdot mol^{-1}$ (data from Müller et al. 1981)

Table 3. Reconstitution of lactic dehydrogenase from pig skeletal muscle after dissociation in guanidine · HCl (0.2 M Na-phosphate pH 7.6, 1 mM EDTA, 5 mM dithioerythritol)[a]

Dissociation			Reconstitution			
c_{Gdn} [M]	c_{LDH} [μg/ml]	$10^{-3} \cdot M_r$	c_{Gdn} [M]	c_{LDH} [μg/ml]	$10^{-3} \cdot M_r$	k_2 [$M^{-1} \cdot s^{-1}$]
0	480	139 ± 2	0	0.3–30	140 ± 3	–
0.8	300	73 ± 10	0.008	3	144 ± 5	$1.5 \cdot 10^4$
1.0	300	80 ± 7	0.01	3 –30	138 ± 5	$1.7 \cdot 10^4$
6.0	300	36 ± 1	0.06	3	140 ± 3	$1.6 \cdot 10^4$

[a] cf. Jaenicke et al. 1981b
[b] Corrected for partial deactivation due to residual guanidine · HCl (Zettlmeißl et al. 1979b)

Association and Enzymatic Activity

A great number of oligomeric enzymes has been reported to require their native assembly structure in order to exhibit full enzymatic function. The second-order steps observed in reactivation kinetics confirm this statement. However, the kinetics do not allow us to specify the active entity (except for dimeric systems). To answer the question of whether intermediates of association are active or not, a number of approaches have been invented; these will be illustrated briefly using lactic dehydrogenase as an example.

Reconstitution Experiments Starting from Dimeric Intermediates. Applying medium concentrations of urea or guanidine · HCl, lactic dehydrogenase has been shown to be partially disassembled to the dimer. Starting reconstitution from this intermediate, the association kinetics turn out to be indistinguishable from the kinetics describing the monomer → tetramer transition (Table 3). Since in this case the tetramer must be formed via the dimer, the dimer → tetramer transition must be rate determining in the overall reactivation (Jaenicke et al. 1981).

Characterization of "Stable Dimers". Lactic dehydrogenase is known to be a "dimer of dimers", which is stabilized in its tetrameric state by an *N*-terminal sequence consisting of 20 amino acid residues. Cleaving off this "arm" by partial proteolysis during reconstitution, generates artificial "stable dimers, which still bind to an NAD-specific affinity column, thus proving the active center of the enzyme to be intact. However, there remains no catalytic activity confirming that the tetramer is the active entity (Girg et al. 1981).

Population Analysis during Reconstitution. To characterize the equilibrium properties of the native and dissociated enzyme, as well as the previously mentioned intermediates, gel-chromatography, sedimentation analysis and turbidity measurements were applied. To monitor kinetic changes of particle distribution during reconstitution, all three methods are unsuitable because of insufficient time resolution and artifacts caused by "wrong aggregation".

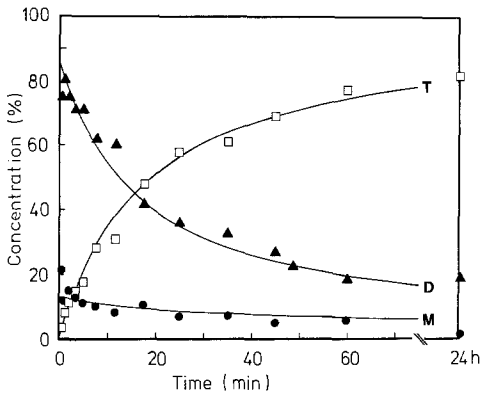


Fig. 8. Chemical cross-linking of lactic dehydrogenase from pig skeletal muscle during reconstitution. Deactivation in 0.1 M H_3PO_4 in the presence of 1.0 M Na_2SO_4, c_{LDH} = 0.56 mg/ml, 20° C. Reactivation in 0.1 M phosphate buffer pH 7.6 (+ 1 mM EDTA), 20° C, by 1 : 200 dilution. Cross-linking with 1% (w/v) glutaraldehyde at c_{LDH} = 2.8 µg/ml at times given. Population analysis from SDS polyacrylamide gel-electrophoresis. Full lines are calculated for tetramers (T), dimers (D), and monomers (M) according to $4\,M \overset{K}{\rightleftharpoons} 2\,D \overset{k}{\rightarrow} T$, with $K = 3 \cdot 10^8\ M^{-1}$ and $k = 3.15 \cdot 10^4\ M^{-1} \cdot s^{-1}$ (cf. Hermann et al. 1981)

Therefore, "snap shot techniques" were devised to follow the kinetics of reassociation. Three methods were successfully applied: time dependent ultrafiltration, hybridization (using different isoenzymes or chemically modified subunits), and chemical cross-linking with glutaraldehyde. The latter approach allows the fast and complete fixation of the native oligomer, as well as the various intermediates on the association pathway without producing artificial interparticle cross bridges (Fig. 8).

Summarizing the foregoing results, reactivation of lactic dehydrogenase is shown to be characterized by fast dimer formation followed by rate determining reassociation to the tetramer which represents the catalytically active species; "structured monomers" and dimers show at most trace activity.

These conclusions cannot necessarily be generalized since each oligomeric enzyme shows individual properties. There are cases where subunits exhibit (partial) activity which may be enhanced or (allosterically) regulated as a consequence of quaternary structure formation. In certain cases subunit assembly is clearly connected with metabolic control in the sense that substrates or products shift a dissociation-association equilibrium according to

$$P_n\,(\text{active}) \rightleftharpoons nP\,(\text{inactive})\,. \tag{6}$$

The inverse situation that disassembly increases or generates activity is rare (if it exists) (Stadtman 1966).

Mechanism of Reconstitution

Kinetic analyses of the concentration dependence of reactivation, renaturation and reassociation prove the overall process of reconstitution to be a superposition of folding reactions with a sequence of second-order association

steps. For the assembly of a native n-mer we may write a general kinetic scheme[3]

$$nM' \xrightarrow{\text{uni}} nM \xrightarrow{\text{bi}} \frac{n}{2} D' \xrightarrow{\text{uni}} \frac{n}{2} D \xrightarrow{\text{bi}} \frac{n}{4} T' \longrightarrow \ldots \tag{7}$$

Thus the strategy in evaluating the mechanism of reconstitution involves the determination of rate limiting steps and the characterization of intermediates in the given scheme. Choosing the regain of enzyme activity as a kinetic parameter an unambiguous kinetic analysis is rendered possible. As indicated by parallel cross-linking, hybridization and reactivation experiments, the recovery of native structure and function may be directly correlated with individual steps in the given reaction scheme. Results for a great number of enzymes may be summarized as follows.

Yields of reconstitution range from 15% to 100%. Processes competing with reconstitution are: (proteolytic) "nicking" of the polypeptide chain, chemical modification of amino acids, proline *cis* $\rightleftharpoons$ *trans* isomerization, kinetic competition of folding and aggregation. If reactivation cannot be accomplished at all syn-translational domain folding or post-translational processing of the nascent polypeptide chain may be assumed. As indicated by sigmoidal profiles and concentration dependent reactivation, consecutive first- and second-order processes are confirmed as rate determining steps in the overall kinetics of reconstitution (cf. Fig. 6). In the case of tetramers, inactive dimers are formed as intermediates in a fast reaction which is followed by the rate-determining tetramerization (parallelled by reactivation) (cf. Fig. 8). The rate of dimer formation may reach the characteristics of diffusion controlled reactions (Groha et al. 1978; Zabori et al. 1980; Seifert et al. 1982). On the other hand, second-order rate constants for tetramer formation vary over a wide range (Table 4). Evidence for active monomers has been obtained using matrix bound subunits (Chan 1970); in only very few examples this finding has been confirmed for the enzymes in solution (creatine kinase, aldolase). In cases where contradictory results were obtained, the matrix may be assumed to "mimick" the subunit interface present in the native quaternary structure; another explanation may be the mobility of the fixed monomers due to the flexibility of the chain molecules forming the matrix.

Ligands may stabilize the nascent structure (as they stabilize the native one), causing a significant enhancement of the yield of reconstitution. For example, the Zn^{2+} ion in the case of liver alcohol dehydrogenase not only provides a Lewis base for catalysis, but also protects "structured monomers" (formed in a first-order precursor reaction) from "wrong aggregation" (Gerschitz et al. 1978; Rudolph et al. 1978). For individual enzymes accelerating effects of ligands on the kinetics of folding and association have been demonstrated (Jaenicke et al. 1980).

3 The scheme considers the fact that, e.g., in the reconstitution of tetramers trimeric intermediates are only insignificantly populated. The essentially irreversible conditions of in vitro reconstitution experiments allow backward reactions to be neglected

Table 4. Reconstitution of oligomeric enzymes according to a consecutive folding-association mechanism: $2A \xrightarrow{k_1} 2B \xrightarrow{k_2} C$ [a]

Enzyme	Source	n	Denaturation	T° C	$10^3 \cdot k_1$ [s^{-1}]	$10^{-4} \cdot k_2$ [$M^{-1} \cdot s^{-1}$]	Mechanism[b]
Alcohol DH (L-ADH)	Horse liver	2	6 M Gdn, pH 7.6	25	7.0	0.16	A
Alcohol DH (Y-ADH)	Yeast	4	6 M Gdn, pH 7.6	25	fast	0.04	A
Aldolase (ALD)	Rabbit muscle	4	pH 2.3	0	1.6	0.18	B
			pH 13	0	1.6	0.18	B
Glyceraldehyde-3-phosphate DH (Y-GAPDH)	Yeast	4	0° C, 5 mM ATP	25	9	7	D
			6 M Gdn, pH 2.3	15	11.0	4.5	C, D
Lactic DH (LDH-H_4)	Pig Heart	4	pH 2.3	20	1.45	0.5	D
			6 M Gdn, pH 2.3	20	1.45	0.5	D
			6 M Gdn, pH 7.6	20	1.45	0.5	D
			6 M urea, pH 2.3	20	1.45	0.5	D
			1.2 kbar, 20 min	20	1.5	0.35	D
			2.0 kbar, 20 min	20	0.35	0.13	D
Lactic DH (LDH-M_4)	Pig muscle	4	pH 2.3	20	fast	2.3	E
			1.3–1.5 kbar ~ 40 min	20	fast	0.5	E, F
			2.0 kbar, 20 min	20	(15)	0.7	D
			6 M Gdn, pH 6.0	20	0.8	3.0	G
Malic DH (m-MDH)	Pig Heart	2	pH 2.3	20	0.65	3.0	D
			6 M Gdn, pH 7.6	20	0.65	3.0	D
			6 M urea, pH 7.6	20	0.65	3.0	D
Pyruvate DH (PDH)	Bac. stear.	$(ABC_2)_{60}$	pH 2.3	20–50	0.02	fast	E
Triose phosphate isomerase (TIM)	Rabbit muscle	2	6.5 M Gdn, pH 7.5	0	19	30	D
Tryptophan synthase (TSase)	E. coli	$\alpha_2\beta_2$	4.5 M Gdn, pH 2.3	12	0.6	fast	E
			2 kbar	10	0.65	>40	E
				10	1.9	>40	F

[a] Reconstitution at pH ~ 7.6 at given temperatures initiated by fast dilution or pressure relaxation; n = number of subunits; k_1, k_2 = first and second order rate constants for folding and association, respectively; DH = dehydrogenase; H_4, M_4 = isoenzymes from heart and skeletal muscle, respectively

[b] Determination of the given rate constants:
A, according to a model comprising reversible Zn^{2+} binding (K_{eq} = 0.17 μm^{-1}) to inactive intermediates;
B, according to $2A \rightarrow 2B \rightarrow C$ with 50% of the final activity at the stage of species B;
C, holoenzyme fully saturated with NAD^+; reconstitution is enhanced by coenzyme and coenzyme analogs;
D, according to $2A \rightarrow 2B \rightarrow C$ with C as the only active species;
E, simple linearization according to first- or second-order;
F, renaturation from regain of intrinsic fluorescence;
G, pre-equilibrium at the monomer level does not involve proline isomerization

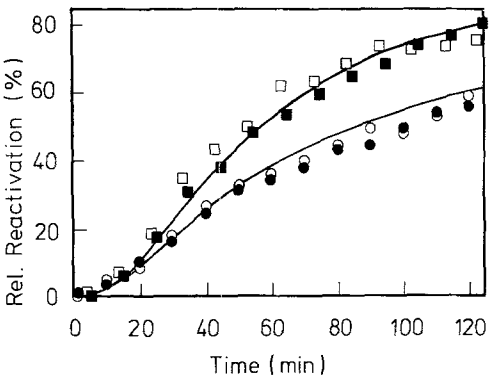


Fig. 9. Specificity in the subunit assembly of lactic dehydrogenase (LDH-H_4) and mitochondrial malic dehydrogenase (m-MDH): Kinetics of reactivation after dissociation in 1 M glycine/H_3PO_4 pH 2.3 (5 min at 20° C), and dilution with 0.2 M phosphate buffer pH 7.6 (+ 1 mM EDTA + 1–10 mM dithioerythritol). Reactivation yields 80 ± 5%. *Open symbols:* separate reactivation of the two enzymes; *closed symbols:* reactivation of mixtures of the two enzymes. (○) 4.05 μg/ml LDH-H_4; (●) 4.05 μg/ml LDH-H_4 plus 2.46 μg/ml m-MDH; (□) 2.46 μg/ml m-MDH, (■) 2.46 μg/ml m-MDH plus 4.05 μg/ml LDH-H_4. Solid lines calculated according to a consecutive folding-association mechanism with $k_1 = 1.4 \cdot 10^{-4}$ s^{-1} and $k_2 = 3 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ for LDH-H_4, and $k_1 = 6.5 \cdot 10^{-4}$ s^{-1} and $k_2 = 15 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ for m-MDH

Specificity of Association

The most interesting facet in discussing the effect of ligands on protein folding is the question of whether or not other proteins interfere with the folding of a given oligomeric enzyme. In order to investigate this problem (which is connected with the specificity of subunit association) synchronous reconstitution experiments with two closely related enzymes – lactic and malic dehydrogenase from pig heart – were performed. As shown by joint incubation of the native enzymes, as well as by joint reconstitution in vitro, after acid dissociation, neither subunit exchange in the native state, nor hybridization after reconstitution occurs (Jaenicke et al. 1981a). Similarly, no changes of the kinetics of reactivation in the absence and presence of the prospective partner of hybridization are observed, thus indicating that no inactive "chimeric" intermediates occur (Fig. 9). Unreconstituted (irreversible) "wrong aggregates" contain both enzymes. Evidently, correct association of oligomeric enzymes arises from highly specific intersubunit binding interactions. In order to maintain specificity of association, the individual subunits must not collide until they have reached a certain level of backbone structure providing the specific recognition sites required for subunit complementarity in a given quaternary structure. In vivo, this requirement is presumably fulfilled by syn-translational folding of the nascent polypeptide chain. The respective association sites must have been selected over evolutionary time for correct assembly, so that no compartmentation is required to guarantee correct quaternary structure formation in vivo (Cook and Koshland 1969).

Table 5. Polymerization of tobacco mosaic virus A-protein (Phosphate buffer $I = 0.05-0.10$, ~ 2 mM EDTA, $c_{TMVP} = 2-56$ mg/ml)[a]

Type of assembly	pH	T_m	$\Delta H°_{cal}$ [kcal/mol]	ΔC_p [cal/K · mol]	$\Delta S°$ [e.u.]	Water release [mol H_2O/ capsomer]
Helical rod	6.45	12.0	12.9 ± 0.3	−350		
	6.75	17.9	12.7 ± 0.3	−350	+40	32
	7.00	21.0	9.8 ± 0.4	− 80		
Double disc	7.50	24.4	6.0 ± 0.1	+150		

[a] cf. Jaenicke and Lauffer 1969; Sturtevant et al. 1981. T_m, the temperature of maximal excess heat capacity (c_{TMVP} ~ 5 mg/ml) equals the turbidimetrically determined transition temperature

Multimeric Systems – Multienzyme Complexes

Successful attempts to achieve reconstitution of complex biological structures prove that there are no qualitative differences to be expected in extending the scope of the foregoing studies towards multimeric systems. A new facet is the possible cooperativity or interdependence of the constituent polypeptide chains in the joint folding and association.

In the case of multimeric systems like the coat protein of tobacco mosaic virus (TMV), identical capsomers are joined together in an endothermic "polymerization" reaction, yielding virus-like particles of varying size. Being completely reversible, this process has been extensively studied as a model reaction for the elementary processes of protein self-organization (Lauffer 1975; Butler and Durham 1977). However, the correlation of the results with specific stages of the self-assembly seems to be complicated by non-equilibrium properties and by the fact that a variety of different assembly structures is obtained, depending on pH, ionic strength, hydrostatic pressure etc. Calorimetric measurements using differential scanning heat capacity calorimetry prove that the endothermic nature persists under all conditions promoting assembly. On the other hand, the change in apparent heat capacity (ΔC_p) is found to change its sign in going from the "double disc" to the "helical rod", in accordance with the reduced flexibility of the protein upon helix formation (Sturtevant 1977; Sturtevant et al. 1981). The entropic origin of the assembly has been experimentally shown to be caused by water release from the polymerizing units in the process of quaternary structure formation (Table 5, cf. Jaenicke and Lauffer 1969).

Multienzyme complexes raise three new questions in connection with the problem of folding and association: (*i*) is there an upper limit of the size where structure formation requires additional information apart from the amino acid sequence of the constituent polypeptide chains; (*ii*) is structure formation in the case of multifunctional systems an "assembly line" process requiring a specific sequence of events; (*iii*) does a neighbouring chain provide a matrix for the folding and association of other components of the multimeric system.

With respect to the first problem, the in vitro reconstitution, e.g., of the *E. coli ribosome* or TMV, has shown that there seems to be no limit with respect to size, even in the case of multicomponent systems. Attempts to reassemble pyruvate dehydrogenase from Bacillus stearothermophilus (which represents the largest multienzyme complex described so far) illustrate this. On the other hand, increased complexity of the system may require an "assembly program" which in certain cases includes "morphopoietic factors" (Kellenberger 1966).

Multienzyme complexes have been found to be accessible to simple dissociation-association experiments proving that no external factors are required for their assembly. The simplest example is the tryptophan synthase bi-enzyme complex ($\alpha_2\beta_2$) from *E. coli* which may be easily reactivated after dissociation and deactivation by acid, guanidine · HCl, high pressure etc. (Groha et al. 1978; Seifert et al. 1982). In this case, the two separate components, α and β_2 contribute each a specific catalytic site providing partial activity with respect to the two-step overall reaction

$$\text{indole-glycerol-P} \overset{\alpha,\,\alpha_2\beta_2}{\rightleftharpoons} \text{indole} + \text{glyceraldehyde-P}\,,$$

$$\text{indole} + \text{L-serine} \overset{\beta_2,\,\alpha_2\beta_2}{\rightleftharpoons} \text{L-tryptophan}\,. \tag{8}$$

"Channeling" of the common intermediate in the enzyme complex increases the catalytic efficiency of the partial reactions by two orders of magnitude, compared to the turnover numbers of the individual subunits.

In contrast to most of the previously mentioned oligomeric enzymes, the time course of reactivation of the β_2 dimer is governed by first-order kinetics due to rate-limiting folding reactions preceding the diffusion controlled assembly of the structured monomers. The overall mechanism of the reconstitution of the bi-enzyme complex must consist of a uni-bimolecular reaction sequence with the association step generating full enzymatic activity.

A similar mechanism seems to govern the reconstitution of pyruvate dehydrogenase from Bac. stearothermophilus (Jaenicke and Perham 1982). The complex ($\mathbf{M}_r \sim 10^7$) contains multiple copies of four different types of polypeptide chains responsible for three component enzyme activities: pyruvate decarboxylase (E1α, E1β), lipoate acyl transferase (E2) and lipoamide dehydrogenase (E3). It consists of a regular pentagonal icosahedron (E2) forming a core to which E1 and E3 are attached such that the substrate (fixed to a lipoyllysyl swinging arm) can be transferred from one active site to the other. Thus both oxidative decarboxylation and regeneration of the prosthetic group are catalyzed within one single entity.

In contrast to tryptophan synthase (which can be easily dissociated and recombined) the pyruvate dehydrogenase complex cannot be reactivated after separating its four constituent polypeptide chains because the decarboxylase is exceedingly labile. However, reconstitution after joint dissociation-reassociation generates relatively high yields of overall activity. The regain of overall activity is again found to be governed by a first-order reaction. Only at very low

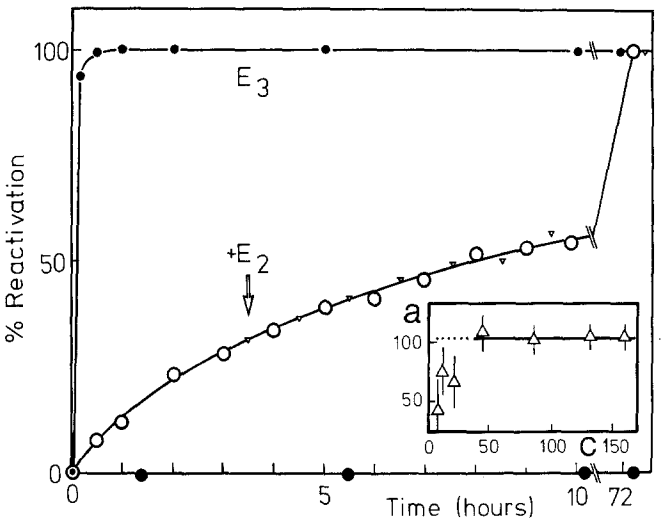


Fig. 10. Reconstitution of the multienzyme complex of pyruvate dehydrogenase from Bacillus stearothermophilus and its constituent enzyme components E2 and E3. Dissociation and deactivation in 1.0 M glycine/H_3PO_4 pH 2.3 (+ 1 mM EDTA + 1–10 mM dithiothreitol, 0° C, 2 min). Reconstitution by dilution with 0.2 M phosphate buffer pH 7.0 (+ 5 mM EDTA + 2 mM dithiothreitol). Reactivation of the PDH complex at 0° C (●) and 53° C (○) at $c_{PDH} \sim 85$ µg/ml. Reactivation of the lipoamide dehydrogenase (E3), and "seeding" (↓) with the lipoate acetyltransferase core (E2) (▼) are not rate-limiting in the reconstitution of overall complex activity. *Insert:* Concentration dependence of the rate of reactivation (initial slope a); c_{PDH} (µg/ml) represents the concentration during reconstitution. Data from Jaenicke and Perham (1982)

enzyme concentration do second-order processes seem to gain influence. Two out of the three partial activities of the complex were investigated separately with respect to their mutual effects on the reconstitution of the whole complex. As shown in Fig. 10 the folding reactions of E2 and E3 are independent of each other. Similarly the various cofactors participating in the catalytic reactions do not affect the kinetics of reconstitution significantly. As in the case of tryptophan synthase intramolecular rearrangements are found to govern the overall reconstitution. This may be interpreted by assuming heterologous interactions of the components of the complex to be involved in a reshuffling process which finally generates catalytic activity.

Concluding Remarks

The acquisition of the three-dimensional structure of proteins consists of sequential folding reactions with defined intermediates. In small proteins the *H*-bonded secondary structure seems to be formed at an early stage followed by folding steps generating the complete tertiary structure. "Folding by parts", i.e., synchronous formation of the secondary and tertiary structures of any part of a polypeptide chain may be assumed to apply only to multi-domain proteins.

To correlate folding and association systematic studies have focussed on oligomeric enzymes applying catalytic activity to follow the kinetics of structure formation. These give information about the folding pathway that is not readily reversible in most cases. Only under conditions generating "structured monomers" can thermodynamic equilibrium be achieved between folded (inactive) monomers and active oligomers. Under these conditions reconstitution may reach 100% native protein.

The major side reaction leading to "wrong aggregates" is easily interpreted as kinetic competition between folding and association. Due to the high specificity of association "chimeric" species are not observed, and even multimeric systems containing different component enzymes show specific assembly.

The given in vitro experiments differ from folding of the nascent polypeptide chain in vivo with respect to the unfolded state which may simply not exist in vivo, at least for proteins beyond a certain size (cf. Baldwin and Creighton 1980). Domain folding during translation of large proteins may prevent the incorrect interactions which are responsible for aggregation and for the slow reshuffling steps during reconstitution in vitro, thus speeding up the folding process significantly.

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