Folding and Interaction of Subunits at the Antibody Combining Site†

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ABSTRACT: The Fv fragment derived from mouse myeloma protein 315, possessing anti-dinitrophenyl (DNP) activity, is composed of two subunits, the peptide chains V_L and V_H. In 8 M urea there is a complete dissociation of V_L and V_H and an approximately twofold increase in the fluorescence emission of Fv with a characteristic red shift of 11 nm. Upon dilution of Fv from 8 M urea into neutral buffer full regain of activity was observed, concomitant with regain of native fluorescence spectrum. The decrease in fluorescence upon dilution from 8 M urea was used to follow the renaturation process of Fv. At relatively high protein concentration (2.5×10^{-6} M) two steps were observed during renaturation: a fast one, which is completed in less than 30 s, and a slower step, which proceeds for approximately 20 min. The fast process represents the refolding and association of V_L and V_H to form an active Fv, whereas the slow step is attributed to the formation of "incorrect" associates between V_L and V_H which slowly reshuffle to the thermodynamically stable active Fv. Indeed, at low protein concentration (1.5×10^{-8} M) only the fast step is observed and renaturation is completed in less than 30 s. The presence of hapten does not affect the rate of renaturation of Fv. Reoxidation of Fv completely reduced in 8 M urea was also found to yield a fully active Fv. Since either V_L or V_H have only one intrachain disulfide bond, reoxidation was performed at high protein concentration (3 mg/ml) in 8 M urea followed by dilution into neutral buffer. This demonstrates that variable domains not only exist in immunoglobulin structure but can also fold correctly independent of the rest of the peptide chains.

The regain of antibody combining site after complete unfolding and reduction of all disulfide bonds provided the strongest evidence that in antibodies, like in other proteins (Anfinsen 1973), the three-dimensional structure is determined solely by the amino acid sequence (Haber, 1964; Whitney and Tanford 1965). It was also shown that the heavy and light chains fold correctly independent of each other (Jaton et al., 1968). In these studies, though substantial amount of activity was regained it never reached the binding activity of the initial antibody. This may be due to the use in these experiments of either Fab or intact antibody which contain several domains and many disulfide bonds. Previously, we reported the preparation of Fv fragment from protein 315, a mouse myeloma protein having high affinity to nitrophenyl ligands (Inbar et al., 1972; Hochman et al., 1973). The Fv fragment has a molecular weight of 25 000 and fully retains the combining site of the intact protein 315. We demonstrated that Fv is composed of two different polypeptide chains, V_L, which is half of the light chain, and V_H, which is a quarter of the heavy chain, each about 110 residues long, and contains one disulfide bond. In 8 M urea Fv dissociates into V_L and V_H, whereas upon dilution from 8 M urea into neutral buffer the binding activity is regained (Hochman et al. 1973).

We studied the process of reoxidation and renaturation of Fv or its subunits after unfolding and reduction in 8 M urea. Reoxidation in 8 M urea and subsequent renaturation by dilution into buffer yield a complete regain of binding activity. This implies that the folding of an immunoglobulin variable domain is independent of the rest of the polypeptide chain.

We also report fluorescence and kinetic studies on the renaturation of V_L and V_H from 8 M urea and their association to form an active Fv. The study shows that the process of renaturation and chain association is very fast, and at the appropriate protein concentration, native conformation and full activity are regained within less than 30 s. The rate of regain of binding activity by renaturation from 8 M urea was not affected by the presence of hapten.

Materials and Methods

Preparation of Fv, V_L, and V_H. Fv was prepared from the Fab′ of protein 315 by pepsin digestion at pH 3.7, as described previously (Hochman et al., 1973). V_L and V_H were separated on DEAE-cellulose in 8 M urea (Hochman et al., 1973). These preparations were either used for the reassociation experiments or desalted on Sephadex G-25 in 0.1 M NH_4HCO_3 and lyophilyzed. The $E_{280}^{1\%}$ of Fv, V_L, and V_H were taken as 15, 10, and 20, respectively (Hochman et al., 1973).

Unfolding and Renaturation Experiments. Fv was incubated for 30 min in 8 M urea, 0.1 M Tris-HCl, pH 9.0, at room temperature, conditions which were shown to dissociate completely Fv (Hochman et al., 1973), and was diluted 20-fold into 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4 (PBS). The fluorescence of the diluted solutions was monitored within 20–30 s after dilution. Some experiments were performed by mixing equimolar amounts of V_L and V_H in 8 M urea. Reduction of Fv or its peptide chains was performed in 0.1 M Tris-HCl, pH 8.2, 8 M urea, and 0.1 M β-mercaptoethanol, at 37 °C for 1 h.

Fluorescence Measurements. Fluorescence measure-

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[1] Abbreviations used are: DEAE, diethylaminoethyl; CD, circular dichroism; CPL, circular polarized luminescence g_{em}, emission anisotropy factor.

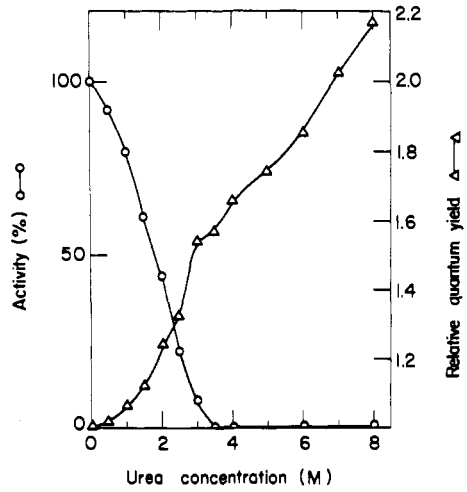

FIGURE 1: Effect of urea on activity and fluorescence of Fv. Fv (10 mg/ml) was diluted 160-fold into solutions of different urea concentrations. After 1 h at room temperature fluorescence spectra were measured, and binding was assayed by fluorescence quenching with DNP-lysine. (Relative quantum yield of the fluorescence emission was calculated from the area of the spectra).

TABLE I: λ_{max} of the Fluorescence of Fv, V_L, and V_H.

	Buffer (native) (nm)	8 M urea (denatured) (nm)
Fv	338	349
V_L	344	350
V_H	342	347

ments were made on a Turner Model 210 recording spectrofluorometer equipped with a constant-temperature circulating bath. Measurements were made at 25 °C in 1-cm path length cuvettes; the excitation wavelength was 280 nm. Changes in fluorescence during renaturation were measured at 375 nm, where the difference between native and denatured protein was large enough (approximately 3.5-fold) to allow significant measurement during the renaturation.

Hapten Binding Analysis. The binding of the hapten N^{ϵ}-DNP-L-lysine to the Fv was followed by the fluorescence quenching method (Eisen, 1964). As will be seen under Results, the fluorescence maximum of Fv in 8 M urea is about twice that of the native protein. During renaturation the fluorescence decreases to the value of native Fv and at any given point in this process, the fluorescence of renaturing Fv is higher than that of native Fv. The measurements of fluorescence quenching during renaturation were performed by adding a twofold molar excess of hapten to the Fv solution and immediately monitoring the fluorescence quenching. Though it is impossible during this fast process to relate directly the fluorescence quenching by hapten to the specific binding of Fv, it is still the simplest way to measure binding activity within 30 s. The ratio between the fluorescence quenching obtained in these measurements and that of native Fv was taken as the fraction of binding activity. When renaturation was performed in the presence of hapten (twofold molar excess over Fv), the fluorescence of these solutions at different time periods was compared with that fluorescence obtained after adding hapten to solutions renatured without hapten.

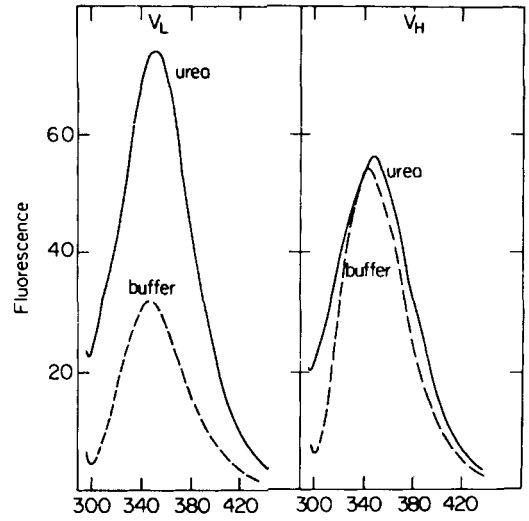

FIGURE 2: Effect of 8 M urea on the fluorescence of V_L and V_H. Protein concentration was 33 μg/ml. Excitation wavelength was 280 nm.

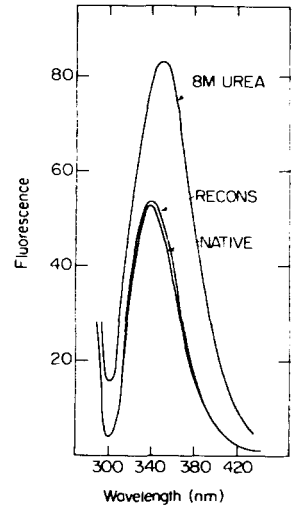

FIGURE 3: Fluorescence emission spectra of Fv in PBS and in 8 M urea. Fv concentration was 66 μg/ml. RECONS, emission spectrum of Fv that was renatured from 8 M urea solution, as described under Materials and Methods. The spectrum was taken 45 min after renaturation.

Results

The Effect of Urea on Fv and Its Component Chains V_L and V_H. Figure 1 shows the effect of increasing urea concentrations on the activity and fluorescence of Fv. Protein fluorescence increases with increasing urea concentration, reaching a twofold increase at 8 M urea, in the fluorescence maximum. On the other hand, the binding activity of Fv is completely lost at 3.5 M urea, and we also found that there is a full dissociation of Fv into V_L and V_H at 3.5 M urea. Thus, Figure 1 indicates that the fluorescence enhancement of Fv above 4 M urea must be due to unfolding of the isolated chains.

The fluorescence emission spectra of V_L and V_H in 8 M urea and in buffer solutions are given in Figure 2. A red shift of 6 and 5 nm in the emission maxima is observed for V_L and V_H, respectively, in 8 M urea (Table I). In the case of V_L in 8 M urea, a 2.3-fold enhancement in the fluorescence maximum is observed, whereas no such change is found in the fluorescence of V_H. Similarly, fluorescence enhancement is also observed for Fv in 8 M urea (Figure 3)

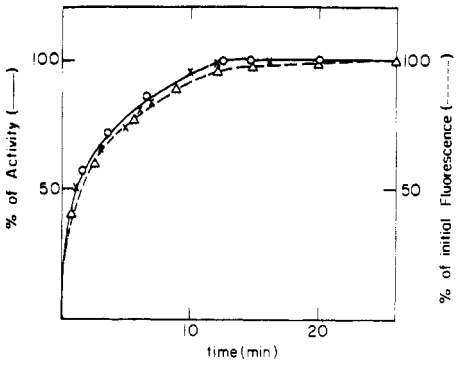

FIGURE 4: Kinetics of renaturation of Fv from 8 M urea. Fv was diluted 20-fold from 8 M urea into PBS to a final concentration of 66 μg/ml and renaturation was followed by decrease in fluorescence at 375 nm (△). Regain of native structure was estimated from the expression $(F_u - F_{obsd})/(F_u - F_n) \times 100$, where F_u, F_{obsd}, and F_n are the fluorescence values in 8 M urea, the observed fluorescence and the native Fv fluorescence at identical protein concentrations. Regain of antibody activity (x) was estimated from fluorescence quenching with the hapten DNP-lysine. At each point hapten was added to a concentration of 2.5×10^{-6} M and the fluorescence was immediately monitored. Activity was calculated as the ratio between fluorescence quenching of renatured Fv and native Fv at the same protein concentration. Hapten effect on the rate of renaturation (○) was tested by diluting Fv from 8 M urea into a solution containing 2.5×10^{-6} M DNP-lysine under conditions identical with the above experiments. The fluorescence values were compared with those depicted by the activity regain curve (x).

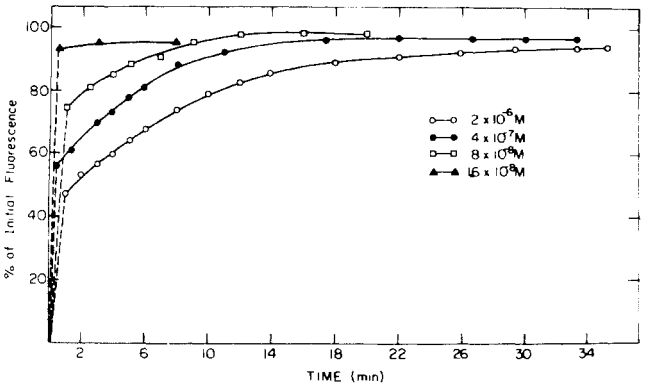

FIGURE 5: Effect of Fv concentration on the rate of renaturation. Fv was renatured from 8 M urea at different protein concentrations and the fluorescence decrease at 375 nm was recorded. The results are expressed as regain of native fluorescence (see Figure 4).

along with a red shift of 11 nm (Table I). Upon renaturation of Fv by dilution from 8 M urea into PBS, there is a decrease, and an appropriate blue shift in the fluorescence maximum (Figure 3). It is noteworthy that the fluorescence spectra of Fv and renatured Fv are practically identical. It is therefore possible to follow the refolding and chain association of Fv by measuring its fluorescence decrease upon dilution from 8 M urea.

Kinetics of Renaturation of Fv from 8 M Urea. Figure 4 shows the rate of Fv renaturation upon 20-fold dilution from 8 M urea into PBS, reaching a final concentration of 66 μg/ml. The renaturation process was followed by measuring the decrease in fluorescence at 375 nm (where the difference is largest, Figure 3), and by measuring binding activity toward N^{ϵ}-Dnp-lysine using the fluorescence quenching method. Several comments may be made with regard to these results. First, the regain of activity follows the same kinetics as the regain of native structure, expressed by the decrease in protein fluorescence. Secondly, the kinetics of renaturation is not influenced by the prescence of hapten in the renaturating solution. Thirdly, the kinetics showed two well resolved steps of renaturation: (1) a very fast step, which cannot be resolved by the regular and manual mixing, comprised about 30% of the regain of native features, and (2) a slow process, which takes place in about 20 min (Figure 4).

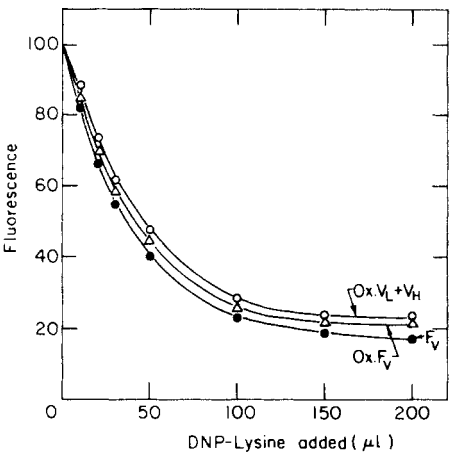

FIGURE 6: Fluorescence quenching analysis of the binding of DNP-lysine by urea-oxidized Fv after renaturation. Fv or V_L were reduced in 8 M urea with β-mercaptoethanol and reoxidized by dialysis against 8 M urea. Fv, or V_L after mixing with equimolar amounts of V_H, were diluted 20-fold into PBS. Final protein concentrations were 50 μg/ml. DNP-lysine was 5×10^{-5} M.

Since the renaturation is expected to involve both a monomolecular process of refolding of each chain separately, as well as their bimolecular association into Fv, we analyzed this process at various protein concentrations. As is shown in Figure 5, the amplitude of the rapid step of renaturation becomes more prominent as protein concentration decreases, and at the lowest protein concentration analyzed (1.6×10^{-8} M), the entire process of renaturation is due to the rapid step only. Since it is very likely that the rate of the bimolecular association will increase with protein concentration, despite our finding that the native conformation at higher concentrations is regained more slowly, we assume that at higher protein concentrations many of the interactions between the chains lead to inactive scrambled associates, which can slowly be converted to the correct more stable native species. At low protein concentration, the high affinity between the correctly folded V_L and V_H is the major force that governs the chain–chain interaction, and the entire process of renaturation takes place in less than 30 s (the time required for mixing and manual measuring of fluorescence).

Oxidation and Renaturation of Completely Reduced Fv. Fv was reduced in 8 M urea, 0.1 M Tris-HCl, pH 8.2, and 0.1 M β-mercaptoethanol at 37 °C for 1 h. Alkylation of a portion of this solution with iodoacetamide, followed by amino acid analysis, showed four carboxymethylcysteines/mol of Fv. Various attempts to oxidize and renature the completely reduced Fv, by removing the urea and mercaptoethanol on Sephadex G-25, or by dilution and dialysis, did not yield an active Fv. Since either V_L or V_H each contain only one disulfide bond, we anticipated that if oxidation would be performed in 8 M urea no "wrong" disulfide bonds could be generated and only the single intrachain disulfide bond would be formed. Reduced Fv (3 mg/ml) was

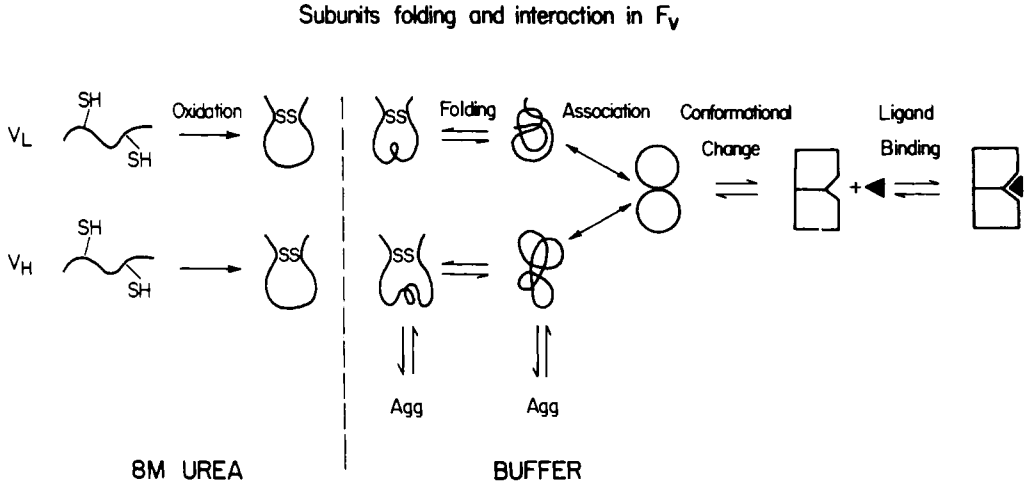

FIGURE 7: Schematic representation of the renaturation of Fv from 8 M urea. The scheme depicts the steps of chain folding and chain association. Only the minimal number of intermediates are given. Agg, aggregates or wrong associates.

dialyzed against 8 M urea to remove β-mercaptoethanol, and during this time it was also reoxidized. A portion of this preparation was alkylated with iodoacetamide and analyzed for CM-Cys after acid hydrolysis. The results did not show any traces of CM-Cys in this urea-oxidized material. In contrast, a sample of Fv, which was alkylated with iodoacetamide before removal of the mercaptoethanol, showed 4.0 CM-Cys/Fv in the amino acid analysis. Dilution of urea-oxidized Fv into 0.1 M NaCl–0.01 M phosphate buffer, pH 7.4, resulted in a complete regain of binding activity by the renatured Fv (Figure 6). Similar experiments were also performed with reduced V_L which was reoxidized in urea and recombined with V_H and diluted into PBS to yield an active Fv (Figure 6).

Discussion

The polypeptide chains of immunoglobulins are comprised of homologous segments, each approximately 100 residues, which fold into structural domains (Edelman and Gall, 1969, Poljak et al., 1972). The noncovalent interactions between domains in trans position (i.e., on different chains) is the basis for the tetrameric structure of the immunoglobulin molecule. Most studies on the interaction between chains were conducted with intact heavy and light chains by dialysis from propionic acid, and the reassociation presumably involved the correct folding and interaction of all domains (four on the heavy chains and two on the light chain). In this report we studied the reoxidation, renaturation, and interaction of the first domain of the heavy chain (V_H) and of the light chain (V_L), which results in the formation of the antibody combining site. Since V_H and V_L are shared by all immunoglobulin classes, the results may be applicable also to chain interaction in the Fv region of all antibodies. Our results reinforce the previously published evidence that the folding of the antibody combining site is entirely dependent on the amino acid sequence (Haber, 1964; Whitney and Tanford, 1965) and is in line with the "thermodynamic" hypothesis of protein folding (Anfinsen, 1973). In our case 100% of the binding activity was regained by reoxidation of completely reduced variable domains of a homogeneous antibody. This was obtained after reoxidation of the V domains in 8 M urea. It is recalled that when such urea-oxidized RNase was prepared, a completely inactive protein with wrong disulfide bonds was obtained (Haber and Anfinsen 1962). Such scrambled inactive RNase was converted to an active RNase by disulfide interchange induced with a low level of β-mercaptoethanol (Haber and Anfinsen, 1962, Givol et al., 1965). In the case of Fv, which has only one intrachain disulfide bond, oxidation in urea after removal of the reducing agent yields a completely active protein. Similarly, urea-oxidized V_L can associate efficiently with V_H to yield an active Fv. The advantage of this procedure is that reduction and reoxidation are performed at high protein concentrations, whereas in other cases of reoxidation and renaturation of proteins high dilution was essential (Epstein et al., 1963).

The complete renaturation of fully reduced Fv provides also a strong support to the domain hypothesis of immunoglobulin structure (Edelman and Gall, 1969). It demonstrates that the V domains not only exist in immunoglobulin structure but can also fold independently of the rest of the peptide chain to form the native structure with full binding activity. Moreover, the folding of V_L and V_H is completely independent from each other. The renatured Fv is identical with native Fv by various criteria, such as fluorescence spectra and full regain of binding activity. We also performed measurements of the circular dichroism of native and renatured Fv which showed identical CD spectra. Several parameters of the interaction between V_L and V_H emerge from this study: (1) There is a good correlation between the renaturation (as followed by the fluorescence of Fv) and the regain of binding activity (Figure 4). (2) The association constant between V_L and V_H is greater than 10^8 M^{-1}, since at a concentration of 1.6×10^{-8} M there is a full and rapid association between V_L and V_H (Figure 5). (3) The ligand, DNP-lysine, does not affect the kinetics of the renaturation and assocation process. This implies that the last step in Figure 7, which is the formation of the combining site itself, is not the rate-limiting step of the renaturation process. (4) The renaturation analyzed in this study by dilution from 8 M urea involves two different processes: (a) chain folding, and (b) chain association.

We assume that the most obvious steps in the renaturation are those depicted in Figure 7. Under our experimental conditions, the first step is the formation of the intrachain disulfide bond in 8 M urea, as is evident by the lack of Cm-Cys after alkylation with iodoacetamide of the urea-oxidized Fv. Dilution from urea to buffer leads to the folding of each chain, which is independent of the other chain. This follows also from our previous results showing that re-

naturation of Fv can be reached either by renaturation of a mixture of chains or by renaturation of each chain separately (Hochman et al., 1973). This is also compatible with the process of biosynthesis, since each chain is made on different polysomes. The third step is chain interaction and association which is accompanied by a conformational change.

The refolding of each chain separately upon dilution from 8 M urea is not easy to study, since no activity is being regained by either V_L or V_H alone. The fluorescence spectra of V_H in 8 M urea and in buffer differ only by a blue shift of 5 nm in the refolded chain. On the other hand, the decrease in fluorescence of V_L upon refolding (Figure 2) can be followed, and was found to be similar in its kinetics to the fluorescence decrease of Fv upon its dilution from 8 M urea. It is clear, however, that the conformation of the peptide chains is different when the chains are separated than when they are combined in Fv. This is reflected in a blue shift of the fluorescence maximum of Fv, as compared with V_L and V_H (Table I), and also in other spectral properties, such as the circularly polarized luminescence (CPL) of Fv and the separated V_L and V_H. Our previous studies (Schlessinger et al., 1975) showed that the CPL spectrum of Fv is not the linear sum of these spectra obtained with V_L and V_H in buffer. The separated chains show very different CPL spectra than intact Fv. While g_{em} of Fv is negative, that of V_H is positive, and that of V_L is zero (Schlessinger et al., 1975). Hence, the changes in fluorescence or in CPL upon recombination of V_H and V_L to form Fv reflect a pronounced change in conformation, leading to the formation of native Fv and its combining site (Figure 7).

The results of renaturation of Fv at very low concentration (1.6×10^{-8} M) indicate that renaturation is completed in a period of seconds. However, at higher protein concentrations the process can be much slower even though the final product will be the same native Fv. We therefore attribute the difference in the rate of reactivation of Fv at different protein concentrations to the formation of "wrong" molecules, inactive associates, etc., which slowly reshuffle to the native active form which is more stable thermodynamically. Although the rate of chain association in other cases of subunit proteins was shown to increase with increasing concentration (Teipel and Koshland, 1971a), there is an effective concentration limit to this behavior, above which the true reaction of renaturation is masked by irrelevant processes such as the formation of aggregates or inactive intermediate forms which are less stable (Figure 7). According to the classification of Teipel and Koshland (1971b) for proteins which fold under thermodynamic control and those that are influenced in their folding by kinetic factors, renaturation of Fv is strictly under thermodynamic control. It is not influenced by the ligand; the eventual extent of renaturation is identical even if the rates are different at different concentration and there is a relatively rapid interconversion of inactive into active forms.

It is significant that at the appropriate protein concentration the rate of renaturation is very fast (probably several seconds) and in the range of renaturation rate of enzyme (Teipel and Koshland, 1971b).

Acknowledgment

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