Alkaline Denaturation of β-Lactoglobulins. Activation Parameters and Effect on Dye Binding Site†

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ABSTRACT: The rate of alkaline denaturation of β-lactoglobulins A and B has been studied at different temperatures and pressures, at pH 9.35. It has been found that pressure greatly increases the rate, and that there is a minimum in the temperature dependence of the rate around 22°. Both the large heat capacity of activation (~1.7 kcal mol^{-1} deg^{-1}) and the large negative volume of activation (~−220 ml/mol) are consistent with the exposure of large hydrophobic regions to the solvent during formation of the transition state. Comparisons of these results with other denaturation processes suggest that the presence of large activation volumes in irreversible denaturations is a good indicator of hydrophobic interactions. In addition, it has been found that denatured β lactoglobulin B is able to bind Bromophenol Blue almost independently of pH. Previous studies had shown that the binding to the nondenatured protein was pH dependent, following closely the "N → R" transition of lactoglobulins. It can then be hypothesized that the binding site, which is exposed to the solvent during the transition, remains "locked" in place after alkaline denaturation.

An earlier work from this laboratory (Waissbluth and Grieger, 1973) was related to the kinetics of the 1:1 binding of Bromophenol Blue to β-lactoglobulin B[1] in a high-pressure temperature-jump apparatus. This process is characterized by two relaxation steps, a fast bimolecular binding followed by a slow isomerization. During the course of that investigation it was found that pressures above 5000 psi produced an irreversible change in the slow relaxation step, with the time constant increasing from 100–300 msec to 1–2 sec, and remaining in that range after the pressure has been released.

A preliminary gel filtration study using Sephadex G-100 showed that the chromatographic pattern obtained after maintaining the protein for 1 hr at 12000 psi was identical with the pattern obtained by Roels *et al.* (1971) after several days at atmospheric pressure. This suggested that the activation volume for the process of alkaline denaturation should be extremely large, and it prompted us to investigate more in detail the effect of pressure on the rate of reaction. As McKenzie and Sawyer (1967) have reported that the rate of denaturation was greater at 3° than at 20° we included temperature as a variable in this study, under the assumption that this should be a characteristic sign of "cold denaturation," with a correspondingly large heat capacity change.

In this report we present data for the rate of denaturation of βLA and βLB at atmospheric pressure between 3 and 41°, and for the rate at 22° between 0 and 6000 psi. The low range of pressures is due only to the fact that above 6000 psi the reaction becomes too fast to be measured by conventional methods. Some effects of denaturation on the binding site for Bromophenol Blue, as shown by kinetic and equilibrium studies, are also reported.

Materials and Methods

βLA and βLB were obtained from Miles Laboratories, and *N*-ethylmaleimide and Bromophenol Blue from Aldrich Chemical Co. Other reagents were of analytical grade, and deionized water was used throughout.

To follow the reaction, we made use of the finding of Roels *et al.* (1971) that the products of the denaturation reaction are insoluble at pH 5.2, and that the rate of decrease of material soluble at pH 5.2 follows first-order kinetics. Solutions were prepared in 0.04 M NaCl–0.01 M glycine at a concentration of 5 mg/ml. As the studies of Roels *et al.* were made in solutions saturated with air, care was taken to maintain the partial pressure of O_2 constant. The buffer was always saturated with air, and the solution was placed, bubble free, in a Teflon cylinder that could be located inside the high-pressure apparatus. As some deviation from first-order kinetics was observed at high conversions, the rates were measured as initial velocities. The reasons for that deviation are unclear, but it is likely that they are related to a reduced concentration of oxygen as the reaction proceeds. This is in agreement with a recent report by Roels *et al.* (1973) who found that denaturation takes place at sensibly lower rates under fully anaerobic conditions. Addition of 2×10^{-3} M *N*-ethylmaleimide almost completely stopped the reaction at high pressures, and this provided further indication that the process taking place at high pressure is the same as the slow process occurring at atmospheric pressure (McKenzie and Sawyer, 1967). Because some experiments ran for several days, 0.002 M NaN_3 was used to prevent bacterial growth. After the required time had elapsed, the solution was brought to pH 5.2 and stored overnight in a refrigerator. The precipitate was centrifuged and redissolved in buffer at pH 9.35. Absorbancy readings were made in both the redissolved precipitate and the supernatant, to verify that the sum of both values remained constant throughout the experiments. A total of some 100 absorbancy readings was taken under different reaction conditions.

The spectrophotometric equilibrium studies were performed as described previously (Waissbluth and Grieger, 1973) and calculations for activation parameters and equilibrium constants were made with linear and nonlinear least-squares subroutines available at the Engineering Computing Labora-

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[1] Abbreviations used are βLA and βLB for β-lactoglobulins A and B. The "N → R" transition of lactoglobulins (Tanford and Taggart, 1961) is a process occurring prior to the process of alkaline denaturation. Consequently, the term "nondenatured protein" used throughout means the R or "reversibly denatured" form of the protein.

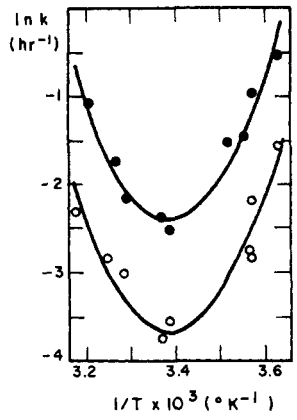

FIGURE 1: Rate of alkaline denaturation of βLA (●) and βLB (○) as a function of temperature at atmospheric pressure.

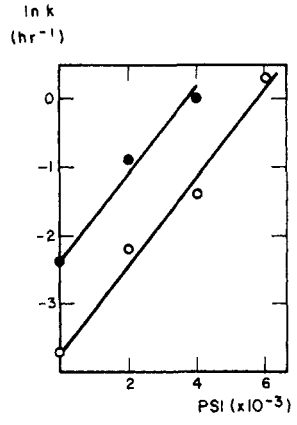

FIGURE 2: Rate of denaturation of βLA (●) and βLB (○) as a function of pressure at 22°.

tory, University of Wisconsin. The spectral red shift that takes place upon binding Bromophenol Blue to denatured β-lactoglobulin is almost identical with that of binding to the nondenatured protein, so that the same wavelength of previous studies (608 nm) was used.

Results

Figures 1 and 2 show the temperature and pressure dependence of the rate of alkaline denaturation of βLA and βLB, and Table I shows the values of the activation parameters for both cases. The similarity of values for both genetic variants is remarkable, and our results are in agreement with those of Roels *et al.* (1971) at 20° and 0.1 ionic strength in that βLA denatures some three times faster than βLB, although in our conditions both proteins denatured at sensibly higher rates (a factor of approximately 4). The precision in the rate constants is on the order of 15%, and further improvements in the accuracy could only be achieved by following the optical rotation as a function of time in a high-pressure apparatus. Nevertheless, the results are quite clear in that the process is characterized in both cases by very high values of $\Delta V^{\ddagger}$ and $\Delta C_p{}^{\ddagger}$, and these values are certainly within an accuracy of 15%. Clearly, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values are strongly temperature dependent. They were found to be in the order of 0 kcal/mol and −85 eu for both proteins around 22°.

TABLE I: Activation Parameters for the Rate of Alkaline Denaturation of Lactoglobulins (0.04 M NaCl, 0.01 M Glycine, pH 9.35, 22°, Atmospheric Pressure).

	$\Delta V^{\ddagger}$ (ml/mol)	$\Delta C_p{}^{\ddagger}$ (kcal mol^{-1} deg^{-1})
βLA	−215	+1.70
βLB	−224	+1.63

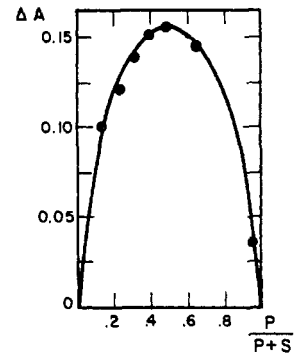

FIGURE 3: Absorbancy readings at constant protein + dye concentration. P = concentration of denatured βLB in moles of monomer; S = molar concentration of Bromophenol Blue; P + S = 6.5 × 10^{-5} M. Same conditions as in Table II.

It is important to note that all kinetic and equilibrium studies at high pressures have to consider the possibility of variations in the degree of ionization of important amino acid residues under high pressure (Waissbluth and Grieger, 1973), because this can lead to misinterpretations of the activation volume. However, in this case the exceedingly large values of $\Delta V^{\ddagger}$ and $\Delta C_p{}^{\ddagger}$, together with the low pressure range and the use of an amino acid as a buffer, preclude the possibility of large errors in the calculated values, in spite of the fact that the rate of alkaline denaturation is pH dependent. A pressure of 6000 psia would produce a shift of approximately 0.1 pH unit. This would be equivalent, from the data of Roels *et al.* (1971), to a change of 15% in the rate, which is negligible when compared with the 50-fold change that we obtain when we increase the pressure to 6000 psia.

Because the alkaline denaturation of βLB had shown some clear effects on Bromophenol Blue binding, some equilibrium studies of this process were done, so as to obtain additional information about the structure of denatured βLB. Figure 3 shows the stoichiometry of the binding and Figure 4 shows the pH dependence of the binding of Bromophenol Blue to nondenatured and denatured βLB. At pH 9.35 the binding to the denatured protein is somewhat weaker, but the main difference consists in the overall shape of the curves. The binding to the denatured protein is almost pH independent, as opposed to

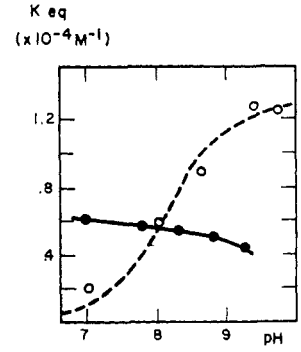

FIGURE 4: Equilibrium constant as a function of pH for the binding of Bromophenol Blue to nondenatured (○) and denatured (●) βLB. I = 0.16, T = 30°.

TABLE II: Thermodynamic Parameters for the Binding of Bromophenol Blue to Nondenatured and Denatured βLB (I = 0.16, Glycine Buffer, 30°, pH 9.35).

	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (eu)
Denatured	−7.3	−5.9	+5
Nondenatured	−8.0	+2.8	+36

the sharp drop in strength of binding observed in the nondenatured protein when going from pH 9 to pH 7. Table II shows the thermodynamic parameters for the binding to the denatured and nondenatured protein (calculated on the basis of mole fraction units), and it can be seen that the driving force for the binding is entropic in nature in one case and mostly enthalpic in nature in the other, with orders of magnitude far beyond the accuracy of the enthalpy calculations (±1.5 kcal).

Discussion

The alkaline denaturation of β-lactoglobulins is a process which is characterized by a transformation of β structure into random structure, and a marked tendency toward polymerization (Roels *et al.*, 1971). The very large $\Delta V^{\ddagger}$ values also indicate that this is a rather drastic process. It is then surprising that the denatured protein still has the ability to bind Bromophenol Blue, with essentially the same spectral red shift and stoichiometry. The binding process is characterized by a two-step interaction. After denaturation, the values of k_1 and

$$P + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PS \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} PS_1$$

k_{-1} remain unchanged (Waissbluth and Grieger, 1973), and this suggests that the binding site is the same before and after denaturation. It is also hypothesized that this site is not obstructed when the denatured protein aggregates, because if this were the case we should observe some change in the time dependence of the fast bimolecular binding step. Moreover, the pH dependence of the association constant indicates that the binding site, which has to be exposed to the solvent during the "N → R" transition in the nondenatured protein (Waissbluth and Grieger, 1973), is irreversibly "locked" on its surface after the alkaline denaturation has taken place.

It is difficult to explain why the driving force for the binding becomes mostly enthalpic after denaturation. These thermodynamic changes have to be related to the observed modifications in the rate constants of the isomerization step ($PS \rightleftarrows PS_1$), but at this stage all one can conclude is that the alkaline denaturation (and further aggregation) of βLB does not obstruct the binding site. The site becomes relatively insensitive to pH variations and remains exposed to the solvent, but with some alterations which are revealed in the conformational changes that follow the binding of Bromophenol Blue to the protein.

The remainder of this discussion will be devoted to hydrophobic bonding, and it is important to summarize some of the highlights of the theory (Frank and Evans, 1945; Kauzmann, 1959; Brandts, 1968), especially in relation to volume and heat capacity changes. It has been well established that the exposure of nonpolar groups to water produces ordered water structures, and this is reflected in a contraction on the order of 6–20 ml/mol of residue exposed to water. At the same time, the tendency of the "icebergs" to melt at higher temperatures

TABLE III: Volume Change for Transfer of a Nonpolar Group from a Nonpolar Solvent to Water at 15 and 25°.[a]

Group	Nonpolar Solvent	$\Delta V_{15°}$	$\Delta V_{25°}$
Benzene	Benzene	−6.1	−6.3
Ethane	Perfluoro-*n*-heptane	−32.8	−32.1
C_2H_4	Alcohols	−2.04	−2.12
C_6H_5	Alcohols	−0.44	+0.22

[a] Values calculated by interpolation from data of Masterton (1954), Gjaldbaek and Hildebrand (1950), Friedman and Scheraga (1965), and American Petroleum Institute (1953).

is shown in a very large partial molar heat capacity change, with values on the order of 50 cal mol^{-1} deg^{-1} per residue exposed to the solvent. These facts have been confirmed by studies in model systems. However, it has also been postulated that at high temperatures the volume changes become less negative because of "iceberg melting," and we feel that this assertion is poorly backed by experimental data. Table III shows volume changes of transfer for some nonpolar compounds, and it can be seen that the temperature dependence of the volume change is at best unclear. On the other hand, Brandts' review (1968) shows that, in model systems, solvated compounds have a smaller compressibility than in a nonpolar medium, thus predicting less strongly negative volume changes of transfer at higher pressures.

If we now turn our attention to the applicability of the model studies to proteins we find two major contradictions. First, studies at high temperature have shown that denaturation is retarded by moderate pressures and accelerated by higher pressures (Brandts, 1968), thus suggesting a larger partial molar compressibility of the activated complex. Second, the reversible equilibrium studies of Brandts *et al.* (1970) on ribonuclease and Hawley (1971) on chymotrypsinogen have shown the rather disturbing fact that while the ΔC_p values are in the order of kcal mol^{-1} deg^{-1} (in agreement with the exposure of hydrophobic groups to the solvent), the ΔV values are surprisingly small if one is to say that hydrophobic contributions are the dominant factor in those processes.

Nevertheless, the results of this work, together with other kinetic data shown in Table IV, indicate that the validity of the hydrophobic model is still clear provided that: (a) we restrict our attention to low temperatures, thus precluding the possiblity of working in the presence of already melted icebergs (in which case hydrogen bonding could become the force that stablizes proteins at moderate pressures); (b) we restrict our attention to the activation parameters of the forward step of denaturation reactions, rather than to overall heat capacity and volume changes. This would preclude the possibility that the $\Delta V^{\ddagger}$ values of forward and reverse reaction rates cancel their contributions to the total volume change associated with the pressure dependence of a reversible denaturation reaction. This does not mean that we neglect the discrepancies found by Brandts *et al.* (1970) and Hawley (1971), because there is obviously something missing in either the forward or the reverse step of these equilibrium reactions which is not contributing to the heat capacity change, but which is contributing to the volume change.

If we analyze Table IV, we can conclude that high $\Delta C_p^{\ddagger}$ values are closely associated with large negative $\Delta V^{\ddagger}$ values. In other words, whenever the heat capacity of activation is large, the activation volume is sensibly more negative than in

TABLE IV: Activation Parameters for Denaturation of Proteins in Aqueous Solution without Denaturants, at Temperatures Below 40°.[a]

Protein	$\Delta C_p^{\pm}$ (kcal mol^{-1} deg^{-1})	$\Delta V^{\pm}$ (ml/mol)	Range of Pressure (atm × 10^3)	Ref
Chymotrypsin	~0	−36 to −40	5.5–6.5	*b*
α-Amylase	~0	−32	6–9	*c*
Trypsin	~0	−19 to −21	6–7.5	*d*
β-Amylase	~0	−33	0–7	*e*
A1-Proteinase	~0	−36	0–10	*e*
Ovalbumin	+1.4	−79 to −92	4.5–5	*f*
Carbonylhemoglobin	+1.5	−68 to −99	4–7	*g*
β-Lactoglobulin A	+1.7	−215	0–0.3	*h*
β-Lactoglobulin B	+1.6	−224	0–0.4	*h*

[a] The references in this table did not mention heat capacity values. Those with 0 heat capacity are the ones which showed a straight line in the Arrhenius plot, and the values for carbonylhemoglobin and ovalbumin were calculated from the kinetic data with the same program used for βLA and βLB. [b] Miyagawa and Suzuki (1962a). [c] Kitamura (1965). [d] Miyagawa and Suzuki (1962b). [e] Kitamura (1966). [f] Suzuki (1958). [g] Suzuki and Kitamura (1960). [h] This work.

the cases where the denaturation process is controlled by forces other than hydrophobic (as judged by the negligible $\Delta C_p^{\pm}$ values). A quantitative analysis of the data obtained in this work, using model compound information on ΔC_p changes, would predict that approximately 32 nonpolar residues are exposed to the solvent during formation of the transition state. If we use model compound data on ΔV changes, some 11–37 groups are exposed to the solvent, and both predictions are in good agreement. It is also important to notice that these very high $\Delta V^{\pm}$ values, probaby the highest measured so far in denaturation reactions, compare very well with the smaller values in ovalbumin and carbonyl hemoglobin, because our data were obtained at a low pressure range. The work of Hamann (1962) on critical micelle concentrations of sodium dodecyl sulfate and of Tuddenham and Alexander (1962) on dodecyltrimethylammonium bromide have shown that the volume change of transfer from a nonpolar medium to water can increase (toward positive values) by 3–11 ml/mol every 1000 atm, so that a total decrease in absolute value of 100 ml/mol for the exposure of more than 10 hydrophobic groups to water between 0 and 4000 atm is entirely within reason. It is then quite likely that the activation volumes for the denaturation of these two proteins, if they could be measured at lower pressures, would be more strongly negative. It is significant that $\Delta V^{\pm}$ for β-lactoglobulin denaturation at pH 5.2 (estimated from plots in the report of Suzuki *et al.*, 1968) is only −80 ml/mol around 3000 atm. Also, interestingly enough, the activation volume in ovalbumin and carbonylhemoglobin decreases strongly with temperature, reaching values around −40 ml/mol at 70°. This would confirm the hypothesis of "iceberg melting" at high temperatures, but it still remains to be proved if that is so in model compounds. Still another piece of experimental evidence which agrees fully with the hypothesis of high $\Delta V^{\pm}$ values at low temperatures and pressures is the work of Kettman *et al.* (1966) on aggregation of poly-L-valyl ribonuclease, which is controlled primarily by hydrophobic bonding of valyl residues. Here the number of residues (22 mol/mol of RNase), the activation volume (+259 ml/mol), and the low temperature and pressure range (39° and 300 atm) fall in line with the values mentioned above for protein denaturation.

Obviously, it is difficult to generalize with only four proteins showing high heat capacities and volumes of activation, but all of them are consistent with the hypothesis that, at low temperatures and pressures, denaturation processes which expose hydrophobic groups to the solvent can be characterized by large heat capacities of activation, large negative volumes of activation, and a tendency of the activation volume to decrease with pressure and temperature. Further work in kinetics of denaturation processes and in the temperature dependence of volume changes in model systems will be necessary to confirm this hypothesis.

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