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Crystalline Leghemoglobin. XV. Effect of Urea on the Conformation of the Slow Component (Lb*a*)

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Soybean ferrileghemoglobin is reversibly unfolded by urea in phosphate buffer, pH 6.5. The unfolding is accompanied by extensive changes in the ultraviolet and visible spectra of the protein, which have been utilized in investigating the equilibrium and kinetics of the unfolding process at 25 °C. The studies were performed on component *a*, Lb*a*, of soybean leghemoglobin and, for comparison, on sperm whale myoglobin. The equilibrium denaturation data showed that the 50 % denaturation point is at 5.15 M urea for leghemoglobin and 7.60 M for sperm whale myoglobin.

The spectrum of denatured leghemoglobin (8.8 M urea) shows an increase in its low spin character, which is assumed to indicate that the heme group is still attached to the denatured protein in the protein concentrations used in this study. The denaturation of ferrileghemoglobin at high urea concentrations was found to be largely reversible upon diluting the reaction mixture. The kinetics of the unfolding reaction for leghemoglobin and for sperm whale myoglobin at pH 6.5 were investigated in the Soret band region. These studies indicate that leghemoglobin is a considerably less stable molecule than sperm whale myoglobin.

Urea denaturation has been extensively used to investigate the forces that maintain the protein molecule in its native form.[1-3] The effect of urea on myoglobin has recently received attention. Schlechter and Epstein [4] have reported a study of the denaturation equilibria of both whale and horse metmyoglobins, and Cann observations on the kinetics of the urea denaturation of metmyoglobin.[5-7] This investigation reports the effect of urea on the slow component of soybean leghemoglobin (Lb*a*). For comparison, some studies were also made on the effect of urea on sperm whale myoglobin.

MATERIALS AND METHODS

Leghemoglobin. The two main components (Lb*a* and Lb*c*) of soybean leghemoglobin were prepared as described previously.[8]

Sperm whale myoglobin was a commercial preparation (Type II) from Sigma Chemical Company (St. Louis, U.S.A.).

Urea, pure, from E. Merck AG (Darmstadt, Germany) was used. It was twice recrystallized from ethanol at 4 °C. Crystals were collected and washed with cold absolute ethanol, dried *in vacuo* and stored *in vacuo* over $CaCl_2$ and $CaSO_4$. Urea solutions were deionized immediately before use by passing the solutions through two ion exchange columns (2 × 8 cm containing 25 ml of Dowex 1 × 2 and 25 ml of Dowex 50 × 2, respectively).

Measurements of absorption spectra. Most of the spectra were measured using a Cary 15 recording spectrophotometer equipped with a jacketed cell holder through which circulated water from a constant temperature bath.

For the absorption spectra measurements at a fixed wavelength a Beckman DU-2 instrument was used without recording attachment.

Procedure for measuring kinetics. A cell containing the buffered urea solution (2.475 ml) was placed in the sample compartment of a Cary 15 recording spectrophotometer. 25 μl of a concentrated solution of Lb*a* or Mb was added to the cell using a cuvette-add-a-mixer. The change in absorption at the Soret band was monitored using the synchronous drive attachment of the spectrophotometer. The denaturation was followed until a very slow linear decline was obtained.

Circular dichroism measurements. Circular dichroism (CD) data for Lb*a* solutions were obtained using a Cary 61 recording spectropolarimeter, which was calibrated with D-10-camphorsulfonic acid. The circular dichroism data were reduced to mean residue ellipticities, $[\theta']\lambda$, using the following formula:

$$[\theta']\lambda = M_0[\theta]/10lc$$

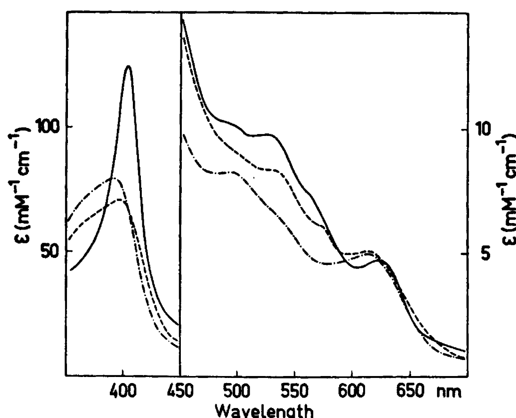


Fig. 1. Absorption spectra of soybean ferrileghemoglobin, Lb*a*, in 0.05 M sodium phosphate buffer, pH 6.5 at 25 °C. (———) native Lb*a*, (– – –) Lb*a* in 8.8 M. For comparison, the spectrum of protohemin IX (– • –) in 8.8 M urea and the same buffer is shown. The actual concentration of Lb*a* and of free protohemin were 7.2×10^{-5} M.

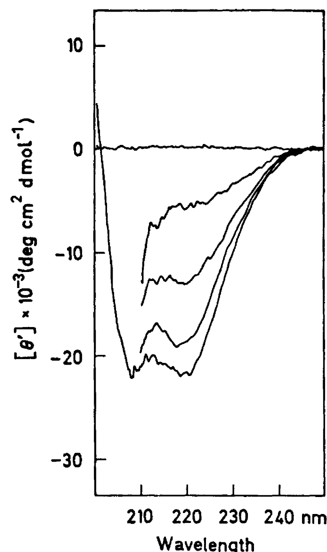


Fig. 2. The effect of urea on circular dichroism spectra of soybean ferrileghemoglobin, Lb*a*, in the spectral region below 250 nm. The measurements were made in 0.05 M sodium phosphate buffer, pH 6.5 at 25 °C, using a silica cell with 1 mm light path. Urea concentrations: 0, 5.0, 6.0, and 8.0 M. The value of mean residue ellipticity increases with increasing urea concentration. The actual protein concentration was 1.02×10^{-5} M.

where M_0, l, c are the mean residue weight, the length of the light path in cm, and concentration of the protein in g/ml, respectively. $[\theta]$ is the ellipticity in degrees recorded on the chart paper. The above formula gives the $[\theta']$ value, expressed in degree cm²/decimol of residues, which satisfies the relationship

$$[\theta]_\lambda = 2.303\ (4.500/\pi)(\varepsilon_L - \varepsilon_R)$$

$$= 3.300(\varepsilon_L - \varepsilon_R)$$

where $(\varepsilon_L - \varepsilon_R)$ is the difference in the molar extinction coefficients for left and right polarized light. The mean residue weight employed for Lb*a* was 106.5.[9]

Pyridine hemochrome was determined according to Paul *et al.*[10]

pH Measurements were carried out at 20 °C with a Radiometer PHM 3 pH-meter, which had been standardized against phthalate and borate buffers. The pH-values given in the experimental section refer to the pH-values of the original buffer.

RESULTS

The absorption spectrum of native Lb*a* and that of the protein in 8.8 M urea at pH 6.5 are shown in Fig. 1. At high urea concentrations spectral changes occur indicating conformational changes at the heme site. The typical ferrihemochrome spectrum with maxima at 530 nm and 565 nm shows that the low spin character of Lb*a* has increased in 8.8 M urea compared to that of protohemin IX in 8.8 M urea (Fig. 1). This is assumed to indicate that the heme moiety is not dissociated, but is attached to the denatured protein to a great extent at the protein concentrations used in this study.

CD spectra of Lb*a* in urea solutions were also measured in the ultraviolet region, as shown in Fig. 2. An increase of the ellipticity in the region of 200 – 250 nm at 8.0 M urea indicates extensive unfolding of the α-helix. It is evident that concomitant changes in the absorption and CD spectra occur with the denaturation by urea. Fig. 3 shows a plot of the fractional absorbance change at the Soret maximum as a function of the urea concentration.

The duration of incubation was found to have an influence on the extinction values; the largest decrease occurred within 30 s at the beginning of the incubation with the values

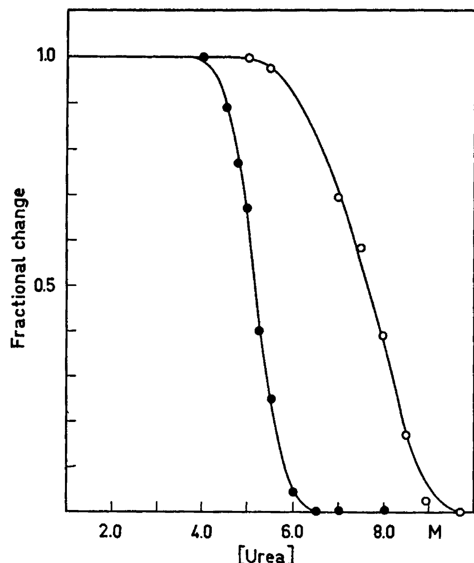


Fig. 3. Fractional absorption change at Soret maximum that occurs on denaturation of soybean ferrileghemoglobin, Lb*a*, (●), and sperm whale ferrimyoglobin, Mb, (○) at different urea concentrations in 0.05 M sodium phosphate buffer, pH 6.5 at 25 °C. The wavelengths used were 403.5 nm (Lb*a*) and 409.5 nm (Mb). The fractional change is equal to $(A_U - A_D)/(A_N - A_D)$, where A_N denotes the absorbance of the native protein, A_D that of the totally denatured protein, and A_U the absorbance at a given urea concentration. The actual protein concentration was 3.34×10^{-6} M.

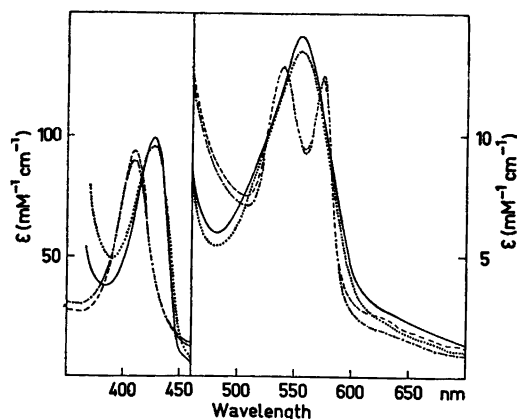


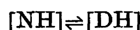
Fig. 4. Spectral properties of soybean leghemoglobin, Lb*a*, after reversal of the denaturation. FerriLb*a* (14.4×10^{-5} M) was denatured at 25 °C for 30 min in 0.05 M sodium phosphate buffer, pH 6.5, containing 8.0 M urea, after which the urea was diluted to 4.0 M with same buffer. (····) shows the spectrum of the reduced form (ferroLb*a*) produced by the addition of a small amount of dithionite. (— · —) represents the spectrum of the oxygen complex, obtained by bubbling air through the solution of the renatured ferroLb*a*. For comparison, the spectra of the native ferroLb*a* (———) and the oxygen complex of the native ferroLb*a* (— — —) in 0.05 M sodium phosphate buffer, pH 6.5, are shown.

decreasing more slowly thereafter. In the case of myoglobin the fast period in the beginning lasted about 10 min. The 50 % denaturation point of Lb*a* was found to be at 5.15 M urea and that of sperm whale myoglobin at 7.60 M urea.

The spectral changes accompanying denaturation of Lb*a* in urea were found to be largely reversible when the urea concentration was reduced by dilution. The recovery of the visible spectrum following denaturation is shown in Fig. 4 for Lb*a* incubated in 8.0 M urea for 1 h prior to dilution to 4.0 M urea. The recovery of the reduced form of Lb*a* was not limited to the return of a native-like spectrum only, the oxygen binding capability of the reduced form was also recovered. This is a strong indication that Lb*a* must have returned to a conformation closely resembling that of the biologically active native form.

The recovery of the intensity of the Soret band upon dilution was found to depend on the length of time a solution was kept in 8.0 M urea before diluting to the lower concentration. If the heme group is dissociated from the denatured protein, the irreversibility may be associated with nonspecific binding to the renatured globin.

These results indicate that an equilibrium exists between the native and unfolded states of ferrileghemoglobin in urea solutions, with the heme groups attached to both forms. It is known to be difficult to distinguish between a simple "two-state" unfolded equilibrium and a more complex, or "multi-state", one. We assume, therefore, a "two-state" mechanism in this particular case, for which the observed transition is



$$[NH] \rightleftharpoons [DH]$$

where [NH] and [DH] represent the heme-

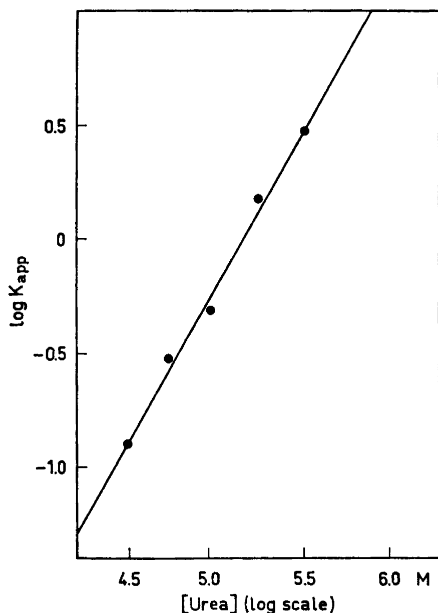


Fig. 5. Plot of log K_{app} *versus* logarithm of the urea concentration. The urea concentration when K_{app} is equal to unity is 5.15 M, and the slope of the line is about 16. The experimental conditions are identical to those in Fig. 3.

protein complex in the native and denatured form, respectively. The equilibrium constant for the denaturation reaction is

$$K_D = [DH]/[NH]$$

and an apparent equilibrium constant can be calculated using an equation of the form [8]

$$K_{app} = A[U]^{\nu}$$

where [U] is the urea concentration and A and ν are constants. A plot of log K_{app} against log [U] is given in Fig. 5. The midpoint of the transition, which occurs when K_{app} is equal to unity, is found to be at 5.15 M urea. The slope was calculated to be about 16.

The effect of urea concentration on the course of denaturation was studied for Lb*a* and, for comparison, sperm whale myoglobin. In Fig. 6A the difference between the extinction coefficient at 403.5 nm at zero time and at time t is plotted against time. Fig. 7A shows a similar plot for myoglobin measured at 409.5 nm. It can be seen that the rate of denaturation of Lb*a* is much faster than that of myoglobin under the same

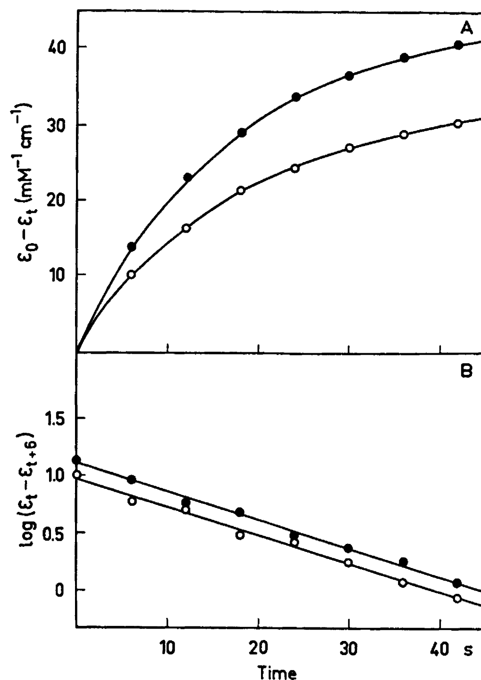


Fig. 6. The denaturation kinetics of soybean ferrileghemoglobin at urea concentrations of 5.25 and 5.50 M measured at 403.5 nm. The experimental conditions were identical to those in Fig. 3. *A*. The kinetics, recorded as differences between the extinction coefficients at zero time, and at the time t, (○) at 5.25 M and (●) at 5.50 M urea. *B*. Guggenheim plots for the denaturation kinetics. The ordinate refers to the logarithm of the difference between extinction coefficients separated by a constant time interval of 6 s, (○) at 5.25 M and (●) at 5.50 M urea.

conditions. Fig. 6B shows a plot according to the Guggenheim method,[11] which eliminates the need for a final absorbance value. The expression used is

$$\ln(A_t - A_{t+\Delta t}) = -(k_{+1} + k_{-1})t + \text{constant}$$

where A_t and $A_{t+\Delta t}$ are absorption values at a time t and $t+\Delta t$, respectively. A Δt of 6 s was chosen. It can be seen that the kinetics at the given urea concentrations are first order as for myoglobin (Fig. 7B).

DISCUSSION

The denaturation behaviour of soybean leghemoglobin (Lb*a*) was studied in urea solutions.

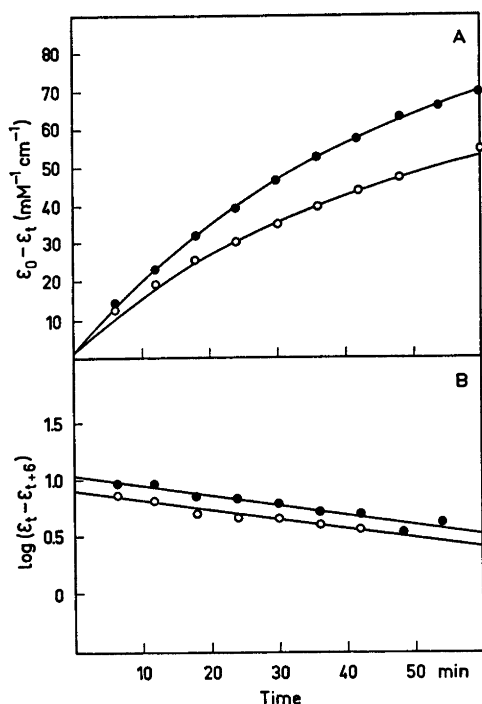


Fig. 7. The reaction kinetics of sperm whale ferrimyoglobin at urea concentrations of 7.5 and 8.0 M measured at 409.5 nm. The experimental conditions were identical to those in Fig. 3. *A*. The kinetics, recorded as differences between the extinction coefficients at zero time and at the time *t*, (○) at 7.5 M and (●) at 8.0 M urea. *B*. Guggenheim plots for the denaturation kinetics. The ordinate refers to the logarithm of the difference between extinction coefficients separated by a constant time interval of 6 min, (○) at 7.5 M and (●) at 8.0 M urea.

Comparative studies have also been performed on sperm whale myoglobin. The equilibrium denaturation data showed that the 50 % denaturation point is at 5.15 M urea for soybean leghemoglobin and 7.60 M urea for sperm whale myoglobin, the latter being close to the values reported in earlier studies.[4,12] The denaturation of the two proteins was found to be reversed by diluting the reaction mixture. It was shown that changes in the absorption spectra and circular dichroism accompanied the denaturation of Lb*a* by urea, but whether this reflects the same structural changes as those responsible for the changes in the heme absorption, or whether it concerns different areas of the molecule, is uncertain.

In the case of heme proteins, including leghemoglobin, the conformational properties of the globin are largely dependent upon the presence of the heme, the globin structure corresponding to a more unfolded state than the heme-complex.[13-17] It is therefore not surprising, that some detachment of the heme groups may accompany denaturation of the protein. However, with the present experimental conditions, it is assumed that no heme dissociation occurs to any large extent during the urea denaturation of leghemoglobin. It is evident that the renaturation of the denatured Lb*a* proceeds more completely when the heme moiety remains attached to the denatured protein. This additionally supports the belief that the heme group has an important role in maintaining the secondary and tertiary structures of the molecule.

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