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Hydrogen–Deuterium Exchange in the Genetic Variants A, B and C of Bovine β-Lactoglobulin

G. B. Ralston

Department of Chemistry, University of Wisconsin, Madison, Wis. 53706, U.S.A.;
present address: Department of Biochemistry, University of Sydney, Sydney, N.S.W. 2006.

Abstract

The hydrogen–deuterium exchange in the β-lactoglobulin genetic variants A, B and C was studied by using an infrared spectrophotometric technique. Between pD 3·1 and 7·6 the exchange behaviour of all three proteins was identical, within the limits of experimental accuracy. Conformation transitions that result in changes in specific optical rotation between pH 4 and 6 for each of the proteins could not be detected by means of the hydrogen exchange measurements, suggesting that this particular conformation change is either very small in terms of the numbers of peptide groups involved, or is limited to rapidly exchanging surface groups. In the pD range studied the exchange of all three proteins was consistent with the EX_2 mechanism.

Introduction

The existence of well-characterized genetic variants of the β-lactoglobulins has prompted a number of comparative studies aimed at determining the effect of amino acid substitutions on the conformation and physical properties of the proteins (Timasheff *et al.* 1966; Alexander and Pace 1971; McKenzie 1971; McKenzie and Ralston 1973). All of the bovine β-lactoglobulin genetic variants are believed to be similar in conformation (McKenzie 1971), although marked differences exist in the association–dissociation behaviour (McKenzie 1971), in the magnitude and pH-dependence of the optical rotatory properties (Timasheff *et al.* 1966) and in the stability towards denaturing agents (Alexander and Pace 1971; McKenzie and Ralston 1973).

The present investigation examines possible differences in conformational dynamics between the three genetic variants A, B and C of bovine β-lactoglobulin, by means of hydrogen–deuterium exchange measurements in regions of pD where differences in optical rotation of the three proteins are known to exist.

Experimental

Materials

β-Lactoglobulins A and B were purchased from Pentex as lyophilized powders, while the C variant was a generous gift from Drs E. T. Adams and E. Kalan. D_2O (98 g-atom of deuterium per 100 g-atom) was obtained from Stohler Isotope Chemicals, and DCl was purchased as a 20% solution in D_2O from the Sigma Chemical Company. Sodium phosphate, sodium borate and NaCl–DCl solutions of ionic strength 0·05, for pD control, were prepared from analytical grade reagents, D_2O and DCl.

Preparation of the Protein Samples

Isoelectric solutions of the three proteins (approximately 2 g/dl) were extensively dialysed against 0·1M NaCl, filtered through washed 0·45-μm Millipore filters and lyophilized in small glass sample bottles of 1 ml capacity as described previously (Hvidt 1963; Ralston 1972). Electrophoresis on 7·5% polyacrylamide gels in 0·03M borate buffer, pH 8·6, confirmed the identity of each protein. No detectable amounts of aggregated material could be found by electrophoresis, nor by means of gel filtration on Sephadex G200 columns (2·5 by 60 cm) in 0·1M NaCl.

Measurement of pD

The lyophilized proteins were dissolved in the original volume of the appropriate D_2O buffers. The apparent pH of duplicate solutions was measured with a Radiometer pH meter, model PHM26, fitted with a Radiometer combination electrode, GK 2301B, and was converted to pD by means of the relationship (Glasoe and Long 1960): pD = apparent pH + 0·4. Duplicate measurements agreed to within 0·05 pH units for a given protein–buffer combination.

Exchange Experiments

The exchange reaction was followed by means of the change in absorption of the amide II band, located at 1545 cm^{-1}, as measured with a Perkin–Elmer double grating infrared spectrophotometer, model 421. This instrument was operated in the scale-change mode, with automatic gain control, and with a fixed slit width of 750 μm. At this setting the spectral band width was 5·2 cm^{-1} at 1400 cm^{-1} and 8·9 cm^{-1} at 1800 cm^{-1}. The instrument room was maintained at a temperature of 25 ± 1°C.

The procedures for initiating and following the exchange reaction and for determining the initial and background absorbances have been described in detail by Ralston (1972), together with methods for calculating the apparent degree of exchange from the amide I and amide II absorbance ratios, and its correction for the effect of the deuteration of the protein.

Results and Discussion

The infrared spectra of the β-lactoglobulin genetic variants revealed the main features described previously (Ralston 1972) with only minor differences. The absorbance of the amide I band increased gradually with increasing degree of exchange, but when correction for this was applied the corrected absorbance of the amide I band remained approximately constant within each experiment.

The background value of the amide II : amide I ratio was 0·05 at pD 1·5 and 0·18 at pD 9·2, slightly higher than the value of 0·15 which was used in a previous investigation (Ralston 1972). The initial ratio, extrapolated to zero time, was 0·47, corresponding to 0·36 when expressed in terms of the absorbance of the fully deuterated protein. Exchange curves for the three genetic variants at a given nominal pD value were very similar. However, in order to take into account the small differences in actual pD, and to test for the possible effects of pD-dependent conformation changes on exchange behaviour, the data have been plotted by the method of Willumsen (1971). In the simple exchange model proposed by Linderstrøm-Lang (Hvidt and Nielsen 1966), any peptide hydrogen may be considered as existing in a conformation shielded from water, or in a partially unfolded conformation and exposed to water. In the exposed conformation the ith peptide hydrogen may exchange with water, with a rate constant k_3 characteristic of the unhindered exchange of model peptides and random coil polyamino acids:

$$\text{Shielded} \underset{k_{2,i}}{\overset{k_{1,i}}{\rightleftharpoons}} \text{Exposed} \xrightarrow{k_3} \text{Exchange}. \qquad (1)$$

The particular limiting case of this model, in which k_1/k_2 is constant, and $k_2 \gg k_1, k_3$, has been designated the 'EX$_2$ mechanism' (Hvidt and Nielsen 1966), and in this mechanism the observed rate of exchange for the ith peptide hydrogen may be written:

$$k_{\mathrm{obs},i} = k_3(k_{1,i}/k_{2,i}). \tag{2}$$

Willumsen (1971) has shown that, if exchange occurs via the EX$_2$ mechanism, then the number (or fraction) of unexchanged hydrogen atoms is a continuous function of k_3t, and data obtained at different pD values or at different temperatures will lie on a continuous curve when plotted as percentage unexchanged hydrogen atoms $v.$ $\log(k_3t)$. On the other hand, if the requirements for the EX$_2$ mechanism are not met (for instance, if k_1/k_2 varies with pD, or if k_3 is not rate limiting), then the Willumsen plot will not be continuous, and data obtained at different pD values will lie on separate curve segments.

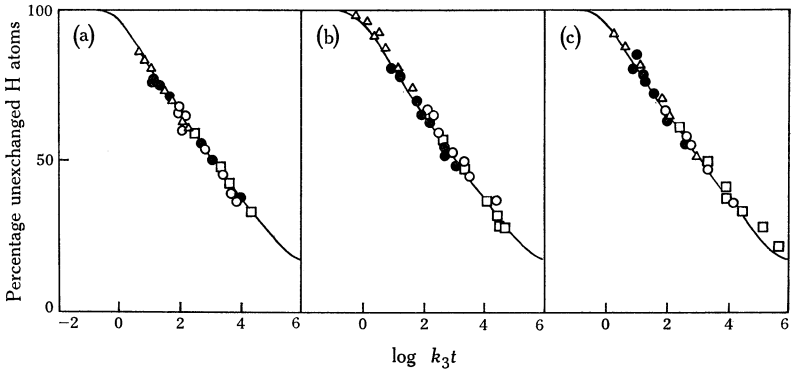

Fig. 1. Willumsen plots for exchange of β-lactoglobulin genetic variants at several different pD values. The solid line is drawn identically in all plots.

(*a*) β-Lactoglobulin A

Symbol	pD	k_3
△	3·9	$10^{0.35}$
●	5·2	$10^{1.0}$
○	6·5	$10^{1.9}$
□	7·4	$10^{2.6}$

(*b*) β-Lactoglobulin B

Symbol	pD	k_3
△	3·2	$10^{-0.1}$
●	4·8	$10^{0.8}$
○	6·5	$10^{1.9}$
□	7·2	$10^{2.4}$

(*c*) β-Lactoglobulin C

Symbol	pD	k_3
△	3·6	$10^{0.15}$
●	4·8	$10^{0.8}$
○	6·5	$10^{1.9}$
□	7·1	$10^{2.35}$

In the present study, poly-DL-alanine was taken as a model of unhindered exchange for the purpose of determining k_3 and its variation with pD (Hvidt and Nielsen 1966). Corrections were made to k_3 to take account of electrostatic effects (Hvidt and Nielsen 1966). Although the use of k_3 data from poly-DL-alanine is an oversimplification, and in general the peptide groups of real proteins will display a spread of values for k_3 (Woodward and Rosenberg 1970), the errors introduced will mainly influence the absolute values of the ratio k_1/k_2, the physical meaning of which is still an open question. For comparative studies of very closely related proteins of similar composition, such as the β-lactoglobulins, the use of k_3 data from poly-DL-alanine would appear to be justified.

The results of the present investigation, when treated as described above, are shown in Fig. 1. The curves for the exchange at different pD values overlapped to

give a single continuous curve for each of the three variants, implying that the observed pD dependence of exchange rate can be accounted for entirely by the pD dependence of k_3. Thus, the exchange of each protein is consistent with the EX_2 mechanism over the pD range 3·1–7·6.

In addition, the curves for the three variants were indistinguishable, as can be seen from Figs 1(*a–c*), in which the solid curve has been drawn identically in each case. Thus, within the limits of experimental accuracy, the hydrogen–deuterium exchange of the three genetic variants of β-lactoglobulin was identical.

No significant change in exchange behaviour of any of the three proteins could be detected in the region corresponding to pH 4–6, in which region pH-dependent optical rotation changes are known to occur in all three variants (Timasheff *et al.* 1966; McKenzie 1971). The changes involved may be too small (either in magnitude of the change or in the number of peptide groups involved) to be detected by means of hydrogen exchange measurements, for which the limit of detection is about 2%. Alternatively, the conformation changes may occur in regions of the protein that exchange too rapidly to be measurable in the high pD conformation (surface groups), or in regions that exchange so slowly that they are inaccessible to measurement in the low pD conformation (deeply buried groups). The most likely explanation is that the conformation transitions are limited to a small number of rapidly exchanging groups on the protein surface. In comparison with the changes involved during the pH 6–9 transitions, or those found on modification of the sulphydryl group (Ralston 1972), the pH 4–6 transition in all three variants is minor in terms of hydrogen exchange behaviour. In addition to the pH-dependent conformation transitions, all of the bovine β-lactoglobulins undergo dissociation from the dimer to the monomer at pH values above and below the isoelectric point, pH 5·2. The A variant, moreover, undergoes association to form a cyclic octamer at low temperatures near pH 4·6 (McKenzie 1971). None of these association–dissociation reactions appears to affect the hydrogen exchange behaviour to a measurable extent. This is the more remarkable because the octamerization of the A variant is enhanced in deuterium oxide (Baghurst *et al.* 1972). Presumably, any effect on hydrogen exchange that is brought about by the association reactions is also small in magnitude and restricted to surface groups.

Already a large number of different studies have indicated the close similarity in conformation of the β-lactoglobulin genetic variants (McKenzie 1971). The results of the present study would strengthen this conclusion, and suggest further that the conformational dynamics of the three proteins in the native state are also remarkably similar.

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