Aggregation of β-lactoglobulin and influence of D_2O

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Abstract The conformational stability of β-lactoglobulin increases in D_2O over that in H_2O. This is concluded from an increase in peak temperature by about 3°C of differential scanning calorimetry (DSC) thermograms and from a decrease in overall aggregation rate. However, effects of pH and salt concentration on the heat-induced aggregation (reaction kinetics, DSC thermograms and aggregate growth) are similar in H_2O and D_2O. This indicates that the mechanism of heat-induced aggregation of β-lactoglobulin is not significantly affected by replacement of H_2O with D_2O.

Key words: D_2O; β-Lactoglobulin; Protein stability; Protein aggregation; Light scattering; Differential scanning calorimetry

1. Introduction

D_2O is often used in nuclear magnetic resonance (NMR) and small-angle neutron scattering (SANS) experiments, aimed at studying protein structure, stability and enzyme kinetics. We have used D_2O as a solvent in SANS experiments to investigate the association and heat-induced aggregation of β-lactoglobulin (β-lg) (to be published). We observed that under given experimental conditions the aggregation in D_2O occurred at a significantly lower rate than in H_2O. In order to determine whether replacement of H_2O with D_2O influences the mechanism by which the heat-induced denaturation and aggregation of β-lg takes place, a comparative study was undertaken. Previous studies already revealed that the conformational stability of some proteins increases in D_2O over that in H_2O [1–8]. This stabilizing effect of D_2O was explained by an increase in hydrophobic interactions and/or an isotope effect on hydrogen bonding [3,5,6].

β-Lg is a globular, heat-sensitive protein and has a molecular mass of 18.3 kDa and a radius of 1.8 nm [9,10]. It is the major protein in whey, the fluid which remains from milk after cheese making. At elevated temperatures (>60°C) the protein denatures and, as a result of conformational changes, subsequently aggregates irreversibly [11]. The heat-induced denaturation and aggregation of β-lg can be described by a simplified reaction mechanism consisting of two consecutive steps: a denaturation step and an aggregation step [12]:

$$\mathrm{B} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{B}^* \quad \text{denaturation} \tag{1}$$

$$\mathrm{B} \text{ or } \mathrm{B}^* + \mathrm{B}^*_x \xrightarrow{k_2} \mathrm{B}^*_{x+1} \rightarrow \ldots \rightarrow \mathrm{B}^*_m \text{ aggregation} \tag{2}$$

An equilibrium between the native state and the (partially) unfolded state of the protein is established in the denaturation step. Unfolding of β-lg is combined with exposure of the free thiol group, which then becomes reactive [13]. After denaturation several irreversible aggregation reactions (Eq. 2) can occur. Around neutral pH and at low ionic strength, mainly disulfide-linked aggregates are formed by chemical reactions through intermolecular thiol group-disulfide bond exchange (propagation) reactions between reactive, denatured intermediates and native β-lg molecules. Thiol group-thiol group (termination) reactions of reactive intermediates are also involved in the formation of these relatively small polymeric particles (size less than 100 nm) [14,15]. At pH values nearer to the isoelectric point and/or at higher ionic strength much larger aggregates are formed via a complex mechanism in which physical bonding through hydrophobic, van der Waals and/or electrostatic interactions plays a major role [16].

We used three complementary techniques to study the heat-induced aggregation of β-lg in H_2O and D_2O under different solvent conditions (pH 6.5/pH 7.0, 0 M NaCl/0.1 M NaCl). The rate of formation of aggregates was followed by measuring the concentration decrease of non-aggregated β-lg as a function of heating time. The enthalpy of the denaturation/aggregation process was probed by differential scanning calorimetry (DSC) and the formation of aggregates was monitored in situ by dynamic light scattering [12,15].

2. Materials and methods

2.1. Chemicals and sample preparation

Chemicals were of analytical grade. Purified β-lg (N1β-lg) was prepared at NIZO from cheese whey, basically following the procedure of Maubois et al. [17], and contained the genetic variants A and B in a nearly 1:1 ratio [15]. The trace of large particles in the sample was removed by centrifugation at $90\,000 \times g$ (sample N2β-lg). D_2O (99.9%) was obtained from Sigma (St. Louis, MO, USA).

β-Lg dispersions were prepared by dissolving β-lg powder in 0 or 0.1 M NaCl solutions that were made using double-distilled H_2O or D_2O. The N1β-lg sample was used for the kinetic experiments and the N2β-lg sample for all other experiments. It was assumed that the trace of large particles did not affect the aggregation kinetics. The protein dispersions were stirred for at least 2 h and the pH was adjusted to the desired value using HCl, DCl, NaOH or NaOD. To correct for isotope effects on the glass electrode and the ionization constants of the ionizable amino acid side chains the pH of protein solutions in D_2O was defined as: pH = pH (meter reading)−0.1 [18]. The dispersions were filtered using a 0.22 μm non-protein-adsorbing filter for the experiments with DSC and the kinetic experiments, and double-filtered using 0.1 μm non-protein-adsorbing filters for the light scattering experiments to remove insoluble materials and dust.

2.2. Differential scanning calorimetry

The thermal behavior of β-lg solutions (63 g/l at pH 6.5 and 7.0 and NaCl concentrations of 0 M and 0.1 M in D_2O and H_2O) was studied using DSC with a Perkin Elmer DSC7 within the temperature interval of 20–110°C at a scan rate of 5°C/min. Approximately 20 mg of

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sample solution was weighed into coated aluminum pans (TA Instruments). As reference the same amount of already denatured/aggregated β-lg solution was used. The peak temperature (i.e. the temperature corresponding to maximum excess heat capacity) was determined from 3–5 replicate runs; it varied by not more than 0.5°C.

2.3. Kinetics of heat-induced aggregation

β-Lg dispersions (9 g/l at pH 7.0 and NaCl concentrations of 0 and 0.1 M in D_2O and H_2O) were heated in test tubes at 68.5°C for different time periods. The tubes were cooled at 0°C for 5 min and the pH was adjusted to 4.7 ± 0.1, at which denatured/aggregated protein precipitates [19]. The denatured/aggregated proteins were sedimented by centrifugation for 30 min at $20\,000 \times g$. The concentration of native β-lg in the supernatant was determined by high-performance gel permeation chromatography (HP-GPC) as described by Hoffmann et al. [15]. Note that β-lg that is denatured (Eq. 1) but not yet aggregated at a certain heating time is determined as native (non-aggregated) protein by this heat-quench method, since the equilibrium step in Eq. 1 shifts to the left upon cooling.

2.4. Light scattering

The average size of the aggregates formed during heating at 68.5°C (9 g/l β-lg at pH 6.5 and 7.0 and NaCl concentrations of 0 M and 0.1 M in D_2O and H_2O) was measured in situ using a dynamic light scattering set-up (90° configuration, wave vector in H_2O and D_2O respectively 0.0187 nm^{-1} and 0.0186 nm^{-1}). Both the time-averaged scattering intensity, $I_s(q)$, and the apparent diameter, d_h, were evaluated as a function of time. The apparent diameter of the particles in solution was calculated from a cumulant fit of the intensity autocorrelation function. For smaller particles (< 100 nm) 90° scattering is in a good approximation proportional to the concentration and molecular mass of the particles. During the heating experiments the total mass concentration is constant and therefore scattering intensity is assumed to increase in proportion to an average molecular mass of the particles [15].

3. Results and discussion

The denaturation/aggregation of β-lg in H_2O was compared to that in D_2O for two different pH values (pH 6.5 and pH 7.0) and NaCl concentrations (0 M NaCl and 0.1 M NaCl). The choice of solvent conditions for this comparative study is based on recent studies in which the effects of pH and NaCl concentration on the aggregation behavior of β-lg in H_2O were studied [12,13,15]. Around neutral pH at low ionic strength the conformational stability of β-lg, the rate of the denaturation/aggregation process at 68.5°C and the size of the aggregates formed appeared to be highly pH-dependent. Further, the reaction rate is at a maximum around 0.1 M NaCl, and, in contrast to the situation without salt added, at 0.1 M NaCl physical bonding is very important for aggregate formation and large aggregates are formed [15]. Consequently, the comparison between H_2O and D_2O is made under solvent conditions which represent extremes in the kinetics of the overall denaturation/aggregation process and in the aggregates formed. The results obtained with DSC, reaction kinetics and light scattering are presented subsequently.

Table 1
Results from DSC experiments (T_p and ΔH) for β-lg in D_2O and H_2O

[NaCl] (M)	H_2O/D_2O	pH	T_p (°C)	ΔH (J/g)
0	H_2O	7.0	73.3	12
0	D_2O	7.0	76.3	13
0.1	H_2O	7.0	73.9	11
0.1	D_2O	7.0	76.8	13
0	H_2O	6.5	75.2	12
0	D_2O	6.5	77.8	12
0.1	H_2O	6.5	76.0	11
0.1	D_2O	6.5	78.6	13

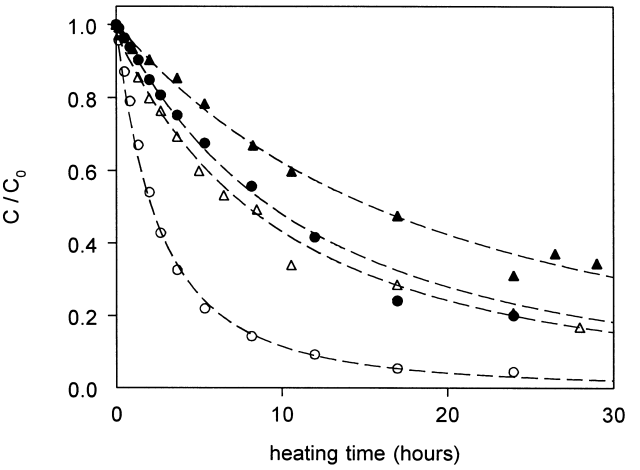

Fig. 1. Fractional concentration of non-aggregated β-lg versus heating time at 68.5°C for initial protein concentrations of 9 g/l at pH 7.0; solvent conditions: △: H_2O, 0 M NaCl; ▲: D_2O, 0 M NaCl; ○: H_2O, 0.1 M NaCl; ●: D_2O, 0.1 M NaCl; the dashed lines represent curves with 1.5 order reaction kinetics fitted to the experimental points [15].

3.1. Differential scanning calorimetry

The stability of β-lg against heat-induced denaturation and aggregation was measured using DSC. Since DSC thermograms are not recorded isothermally but at a given (relatively high) heating rate, they mainly probe the conformational changes in the denaturation reaction [20,21], as shown by Hoffmann at neutral pH [22]. In Table 1, the peak temperature, T_p, and the enthalpy change, ΔH, determined from DSC thermograms, are given for different β-lg solutions with protein concentrations of 63 g/l. It was found that T_p at pH 7.0 is approximately 3°C higher in D_2O compared to H_2O; at pH 6.5 this difference in T_p is somewhat less (2.6°C). Thus, the DSC measurements show a significantly increased conformational stability of the protein in D_2O over that in H_2O, as was already found for some other proteins [1–8]. Addition of 0.1 M NaCl to the medium increased T_p by 0.5–0.8°C at both pH values in D_2O and H_2O. This indicates that NaCl also has a stabilizing effect on the conformation of native β-lg and that the effect is similar in H_2O and D_2O. ΔH of the transition of β-lg is not significantly influenced by D_2O, as has been found before for ribonuclease [4].

3.2. Kinetics

In Fig. 1 the fraction of non-aggregated β-lg is plotted as a function of heating time at 68.5°C for β-lg solutions of 9 g/l in D_2O and H_2O (in the presence and absence of NaCl). The reaction rate of the overall denaturation/aggregation process decreases if H_2O is replaced by D_2O and increases if 0.1 M NaCl is added to the medium. The lower reaction rate in D_2O with respect to H_2O agrees with the increase in conformational stability, and thus the reduced denaturation rate, as observed with DSC (Table 1). The increase in reaction rate by addition of 0.1 M NaCl seems to disagree with the observed increase in T_p. The overall reaction rate at 68.5°C, however, is determined by both the denaturation and the subsequent aggregation of β-lg (see Eqs. 1 and 2). As seen before [12], NaCl

stabilizes the protein and reduces the denaturation rate (Eq. 1), but stimulates the rate of the aggregation reactions (Eq. 2) by screening the charges of the protein, and, consequently, the overall reaction rate is higher.

3.3. Light scattering

Light scattering experiments revealed that upon heating 9 g/l β-lg solutions at 68.5°C without NaCl added, the apparent diameter, d_h, first increases rapidly and then reaches a more or less constant value at pH 6.5 and 7.0, in both D_2O and H_2O (Fig. 2). In D_2O the plateau value for d_h is reached at a later time than in H_2O, due to the lower overall reaction rate. Combining the kinetics of particle formation with the rate of conversion of native β-lg (Fig. 1), it follows that under these conditions polymer particles are formed that, upon heating, grow not in size but in number concentration. These results are in keeping with earlier measurements in H_2O [14]. The plateau value for d_h is smaller at pH 7.0 than at pH 6.5, both in D_2O and in H_2O. If the pH increases from 6.5 to 7.0 more reactive intermediates (i.e. aggregates with an exposed thiol group) are present in the initial stages of the reaction caused by an increase in the denaturation rate in Eq. 1 (see Section 3.1) and an increase of the reactivity of the thiol groups [12,13]. This leads to the formation of more but smaller disulphide-linked aggregates and a higher aggregation rate in Eq. 2 [22]. In D_2O both the overall reaction rate (Fig. 1) and the size of the polymer particles (Fig. 2) are smaller compared to H_2O. This is due to a lower denaturation rate in D_2O (see Section 3.1) and to differences in physical properties between H_2O and D_2O (i.e. viscosity, polarity, pK_a and reactivity of thiol groups [23]), which will affect the propagation and termination reactions.

In Fig. 3, d_h (Fig. 3A) and the scattering intensity, I_s (Fig. 3B), are shown for 9 g/l β-lg at pH 6.5 and 7.0 in D_2O and H_2O in the presence of 0.1 M NaCl. When NaCl is added, the aggregates continue to grow in size as a function of heating time, in contrast with the situation without NaCl (Fig. 2). This is caused by an increased contribution of physical aggregation by screening the charges of the protein and a decreased solubility of the protein particles ('salting-out' effect) [12,24]. A lag phase is observed, followed by a second phase with clear growth of particle size. The 90° scattering data sustain the observations from dynamic light scattering in that they show a sudden increase in scattering intensity, which can only be attributed to rapid growth of clusters. The lag phase is longer in D_2O than in H_2O, corresponding to the lower overall reaction rate in D_2O (Fig. 1). After the lag phase particle size and scattering intensity as a function of the logarithm of heating time increase in the same way in D_2O and H_2O, at both pH 6.5 and pH 7.0. In D_2O the same type of aggregates are formed as in H_2O, but at a reduced rate. In both D_2O and H_2O the lag phase is longer at pH 6.5 than at pH 7.0, due to a lower overall aggregation rate at pH 6.5 [12]. At this pH larger aggregates are formed than at pH 7.0, which is also seen under the conditions where no salt is added (Fig. 3A).

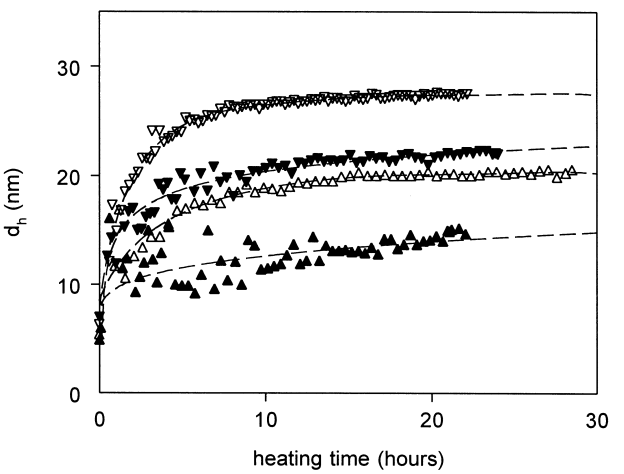

Fig. 2. Hydrodynamic diameter against heating time at 68.5°C for β-lg solutions of 9 g/l without added NaCl; solvent conditions: △: H_2O, pH 7.0; ▲: D_2O, pH 7.0; ▽: H_2O, pH 6.5; ▼: D_2O, pH 6.5; the dashed lines are added to guide the eye.

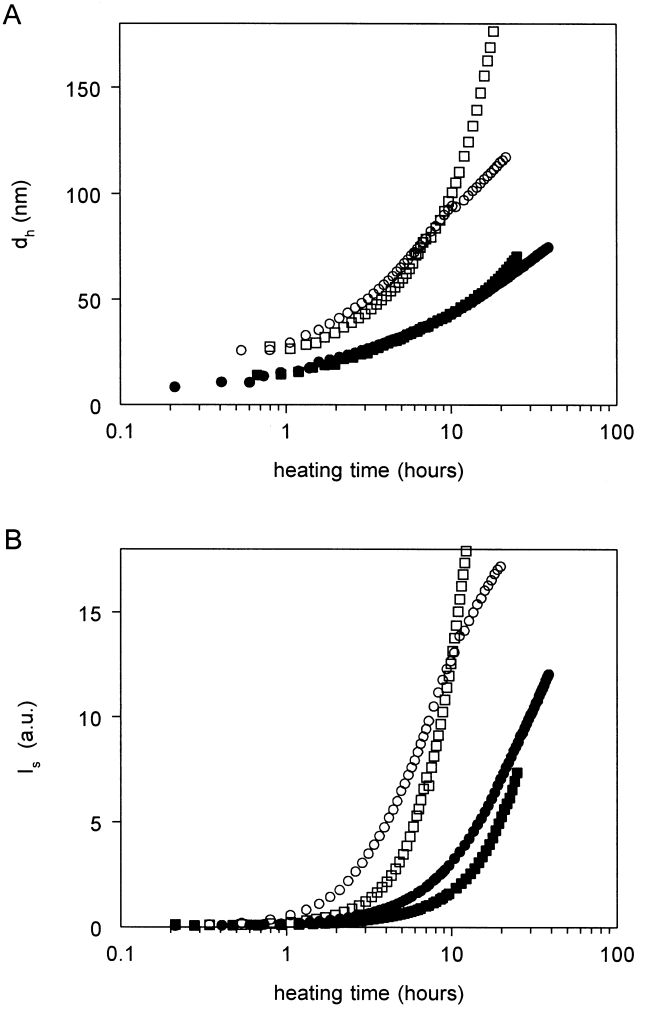

Fig. 3. Hydrodynamic diameter (A) and scattering intensity (B) versus heating time at 68.5°C for β-lg solutions of 9 g/l at 0.1 M NaCl; solvent conditions: ○: H_2O, pH 7.0; ●: D_2O, pH 7.0; □: H_2O, pH 6.5; ■: D_2O, pH 6.5.

3.4. General discussion

The heat-induced aggregation of β-lg can be described with a reaction mechanism consisting of two steps: a denaturation equilibrium followed by irreversible aggregation reactions. From this study it can be concluded that the heat-induced denaturation/aggregation of β-lg is affected by replacement

of the solvent H_2O with D_2O through the stabilizing effect of D_2O on the conformation of β-lg. This follows from the increase in peak temperature of DSC thermograms by about 3°C and is confirmed by the decrease in overall conversion rate of β-lg (at 68.5°C). Variation in pH (pH 6.5 and 7.0) and NaCl concentration (no NaCl and 0.1 M NaCl) results in the same trends with respect to reaction kinetics, DSC thermograms and formation of aggregates in both D_2O and H_2O. Furthermore, it can be concluded that the mechanism of heat-induced denaturation and aggregation of β-lg is not significantly affected by replacement of H_2O with D_2O. Only the absolute value and the ratio of the rates of the different reaction steps are influenced by D_2O.

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