

Low resolution NMR spectroscopy: a tool to study protein denaturation

I. Application to diamagnetic whey proteins

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SUMMARY. A method using low resolution NMR spectroscopy is described for investigating whey protein thermal denaturation. The method is based on measuring at 20 °C changes in water proton transverse (T_2) relaxation parameter following the denaturing treatment. This parameter is shown to be sensitive to protein denaturation and not to other phenomena such as gelation. Examples are given for the qualitative study of protein thermal denaturation in whey protein concentrate, β -lactoglobulin, α -lactalbumin, bovine serum albumin and immunoglobulins aqueous solutions and for the quantitative determination of thermal denaturation in whey protein concentrate solutions.

The functional properties of a food material are essentially determined by its protein function. It is controlled by the molecular structure of the proteins (Zadow, 1986). Denaturation with complete unfolding is of interest for the food scientist as this may arise during thermal processing routinely used in the food industry.

Pulsed NMR spectroscopy has been applied to study the state of water in protein–water systems. In all homogeneous protein samples (Lambelet *et al.* 1988; Maquet *et al.* 1984; Oakes, 1976*a, b*; Goldsmith & Toledo, 1985; Lelièvre & Creamer, 1978; Rydzy & Skrzyński, 1980, 1982; Blicharska & Rydzy, 1979; Mahdi, 1980; Pumpernik *et al.* 1975; Richardson *et al.* 1986) except myosin-containing samples (Nakano & Yasui, 1979; Yasui *et al.* 1979), the water proton transverse (T_2) and longitudinal (T_1) relaxations have been reported to be single exponential processes. Such relaxations were interpreted in terms of a two-state model (Zimmerman & Brittin, 1957) with a rapid exchange between a small number of water molecules tightly bound to the protein and normal bulk water (Lambelet *et al.* 1988; Oakes, 1976*a, b*; Richardson *et al.* 1986). On the other hand, the transverse (Lambelet *et al.* 1988; Oakes, 1976*b*) or the longitudinal (Goldsmith & Toledo, 1985) relaxation time was shown to shorten during the heating of the sample. On this basis denaturation as well as gelation of protein solutions were studied by pulsed NMR spectroscopy using either the T_1 or the T_2 relaxation probe, although the T_2 relaxation probe has been shown to be a more sensitive probe for this purpose (Oakes, 1976*b*).

Thermal denaturation of proteins has been studied by monitoring the transverse or the longitudinal relaxation time during heating (Goldsmith & Toledo, 1985; Rydzy & Skrzyński, 1980; Blicharska & Rydzy, 1979; Pumpernik *et al.* 1975). However, relaxation times being temperature dependent, such a method has the

disadvantage of measuring not only the denaturation but also the pure effect of temperature on the relaxation times. To avoid such interference, we propose for the study of protein thermal denaturation by NMR spectroscopy a method in which the measurements are always effected at the same temperature. The present paper describes this new NMR approach and reports the results obtained by this technique in the case of thermal denaturation of diamagnetic whey proteins.

MATERIALS AND METHODS

Materials

An industrial whey protein concentrate (WPCI) was prepared from sweet cheese whey (Jost *et al.* 1986). The spray-dried product has a protein content (Kjeldahl, $N \times 6.38$) of 82.5% and a Ca content of 0.70%. An undenaturated whey protein concentrate (WPCL) was prepared in the laboratory by ultrafiltration of raw sweet cheese whey. The lyophilized product has a protein content (Kjeldahl, $N \times 6.38$) of 87.4% and a Ca content of 0.29%. β -Lactoglobulin (β -lg) was prepared from sweet whey using the spherosil procedure (Meiller & Mirabel, 1975). The product contained 82.8% protein (Kjeldahl, $N \times 6.38$) and 0.29% Ca. α -Lactalbumin (α -la) (L-6010, lot no. 52 F-80751) and bovine serum albumin (BSA) (A-0281, lot no. 126 F-9350) were obtained from Sigma Chemical Co. (St Louis, MO, USA). The protein concentration (gas chromatography, $N \times 6.38$) was 93.3% (this product might contain traces of $(NH_4)_2SO_4$) and 93.9% respectively, and the Ca content 350 and 195 ppm respectively. The BSA was essentially free of fatty acid. The immunoglobulins (IgG) were prepared from defatted colostral milk by acid precipitation of the casein and salting out with $(NH_4)_2SO_4$. After dialysis, the product was purified by ion exchange chromatography (DEAE-cellulose). The lyophilized product has a protein content (gas chromatography, $N \times 6.38$) of 84.9% and a Ca content of 51 ppm. 2-Mercaptoethanol (analytical grade) was purchased from E. Merck (D-6100 Darmstadt, FRG).

Sample preparation

Whey protein aqueous solutions (sols) were obtained by mixing at room temperature the protein with distilled water to give defined concentrations of proteins between 4 and 20% (w/v). After adjusting the pH to 7.0 with 0.1 M NaOH the mixtures were degassed. To study the influence of pH on denaturation, additional sols were prepared by solubilizing 12% (w/v) β -lg in distilled water and adjusting the pH to levels between 3.0 and 8.0. The sols were then introduced into NMR tubes and the tubes sealed.

Denaturation was achieved by heating the sample for 30 min at defined temperatures between 20 and 100 °C. Denaturation was investigated after each thermal treatment by measuring T_2 water proton relaxation time in samples thermostatted at 20 °C for 30 min.

NMR measurements

NMR measurements were run on a Minispec PC20 (Bruker Physik AG, Karlsruhe, FRG) at a frequency of 20 MHz. The proton transverse magnetization decay curve was determined by sampling 169 echoes stemming from a Carr-Purcell-Meiboom-Gill pulse sequence. T_2 relaxation times were calculated from the spin-echo decay curve as previously described (Lambelet *et al.* 1988).

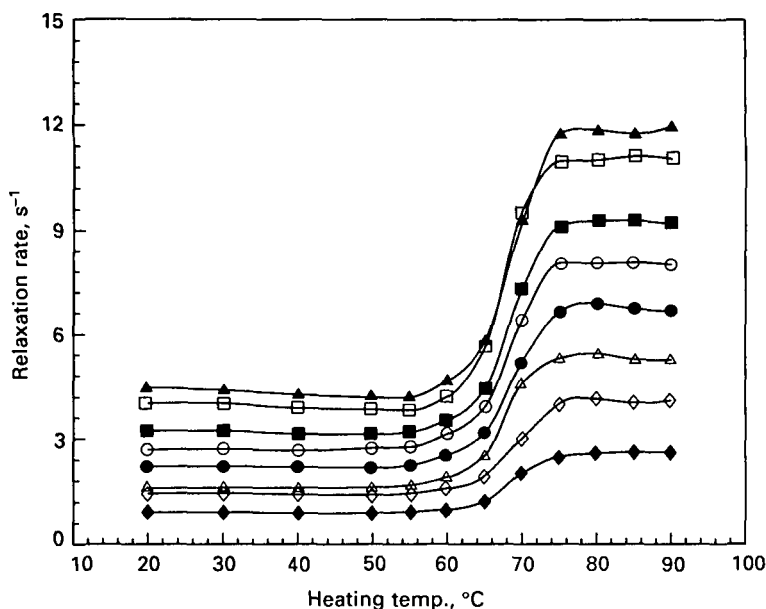


Fig. 1. Water proton T_2 relaxation rates in an industrial whey protein concentrate solution (pH 7.0) heated at various temperatures for 30 min. Protein concentrations: \blacklozenge , 4.0%; \diamond , 5.9%; \triangle , 7.9%; \bullet , 9.9%; \circ , 11.9%; \blacksquare , 13.9%; \square , 15.8%; \blacktriangle , 17.8%.

Determination of protein denaturation (Rowland, 1938)

Total N was determined according to the Association of Official Analytical Chemists' official method (AOAC, 1980). The non-protein nitrogen (NPN) was determined by measuring the N content (AOAC, 1980) in the filtrates after precipitation with 12% trichloroacetic acid. Whey protein nitrogen (WPN) was calculated by subtracting NPN from non-casein nitrogen measured in filtrates after precipitation of samples at pH 4.6 with acetate buffer. The percent denaturation was calculated by comparison of the WPN of heat-treated sample with WPN of raw sample:

$$\% \text{ Denaturation} = \frac{\text{WPN}_{\text{raw}} - \text{WPN}_{\text{heated}}}{\text{WPN}_{\text{raw}}} \cdot 100$$

RESULTS

Water proton T_2 relaxation in all whey protein samples investigated, e.g. in untreated and heated samples, could be analysed in terms of a single exponential characterized by a relaxation time T_2 . The corresponding relaxation rates ($1/T_2$) showed dependence on the temperature of the thermal treatment applied to the sample. After complete thermal denaturation, the relaxation rates were not modified during a second heating of the sample in the same temperature range.

WPCI-based samples

For all concentrations of WPCI between 4 and 18%, the curves relating the water proton transverse relaxation rates to the heating temperature of the sample were similar (Fig. 1): no change in relaxation rate was observed after heating up to 55 °C. However, a sharp increase proportional to protein concentration resulted from

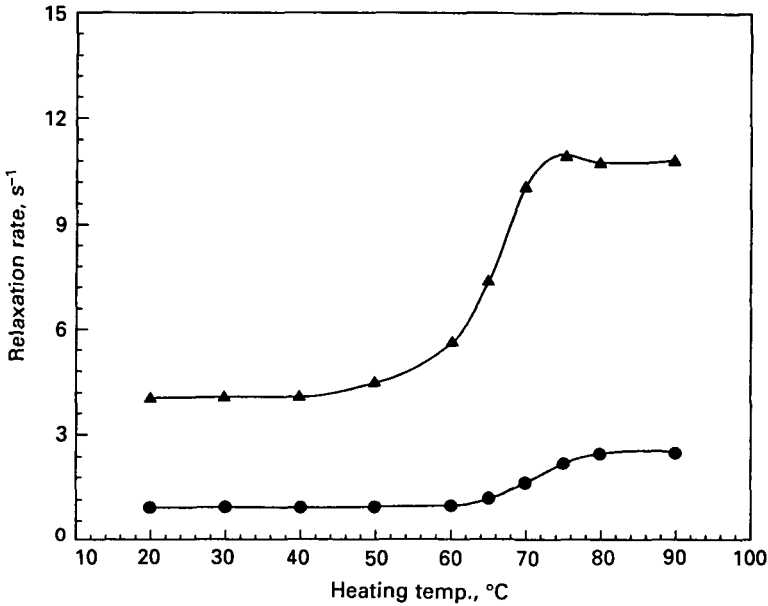


Fig. 2. Water proton T_2 relaxation rates in β -lactoglobulin solution (pH 7.0) heated at various temperatures for 30 min. Protein concentrations: ●, 4%; ▲, 18%.

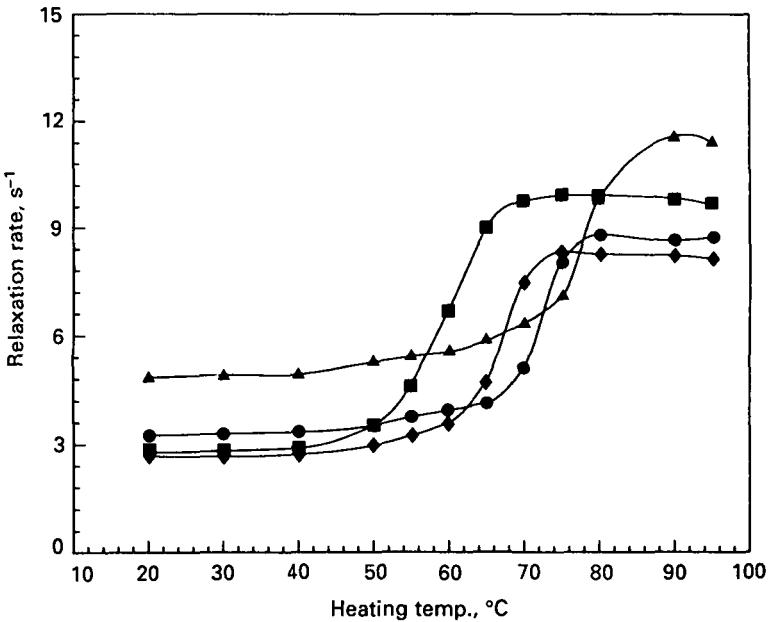


Fig. 3. Influence of pH on the water proton T_2 relaxation rates in 12% β -lactoglobulin solutions heated at various temperatures for 30 min. pH levels: ▲, 3.5; ●, 6.0; ◆, 7.0; ■, 8.0.

thermal treatment in the temperature range between 55 and 75 °C, and no further change was recorded following heating to 90 °C.

β -Lg-based samples

For β -lg samples with protein concentration between 4 and 18%, the functions linking the water proton T_2 relaxation rates to heating temperatures were similar to corresponding functions measured in WPC samples. Heating β -lg samples at pH 7.0

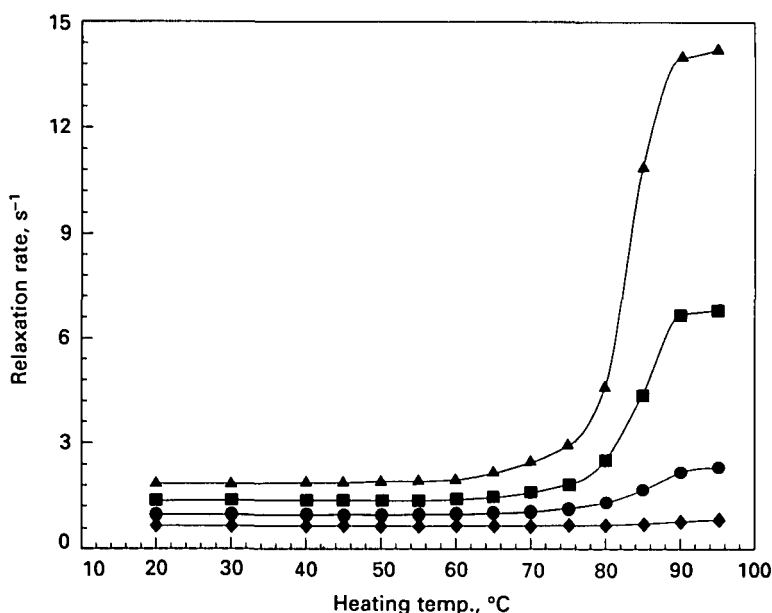


Fig. 4. Water proton T_2 relaxation rates in α -lactalbumin solution (pH 7.0) heated at various temperatures for 30 min. Protein concentrations: ◆, 4.4%; ●, 8.7%; ■, 13.1%; ▲, 19.9%.

below 40 °C did not influence T_2 relaxation rates (Fig. 2). An increase in relaxation rates, proportional to protein content, was observed following heating between 40 and 80 °C. The exact temperature range at which this increase occurred was slightly dependent on the protein content. Thus, it took place between 60 and 80 °C for the 4% protein sample and between 40 and 75 °C for the 18% protein sample (Fig. 2). No modification of relaxation rates was observed during further heating up to 90 °C.

The influence of pH on T_2 relaxation rates measured in 12% β -lg aqueous solutions during thermal treatment is presented in Fig. 3. The main characteristics of the function linking relaxation rates to heating temperatures were not modified in the pH range 3.5 to 8.0. However, the temperature range where an increase in relaxation rates was observed was dependent on the pH of the solution. Dropping this pH from 8.0 to 3.5 resulted in an increase in this temperature range from 40–65 to 60–85 °C (Fig. 3).

α -La-based samples

The water proton T_2 relaxation rates in 4% α -la solutions did not change during 30 min heating up to 95 °C (Fig. 4). Significant increases in T_2 proton relaxation rates were however observed during heating between 80 and 90 °C for 8% protein and between about 70 and 90 °C for 13 and 20% protein samples. Heating above 90 °C did not further modify these relaxation rates (Fig. 4). For samples with protein concentration greater than 4%, the increase in relaxation rates was proportional to the protein level in the sample.

BSA-based samples

For protein concentrations between 4 and 17%, the T_2 relaxation rates in BSA solutions did not change on thermal treatment at temperatures between 20 and 45 °C (Fig. 5). Between 45 and 60 °C a sharp increase in relaxation rates, proportional to

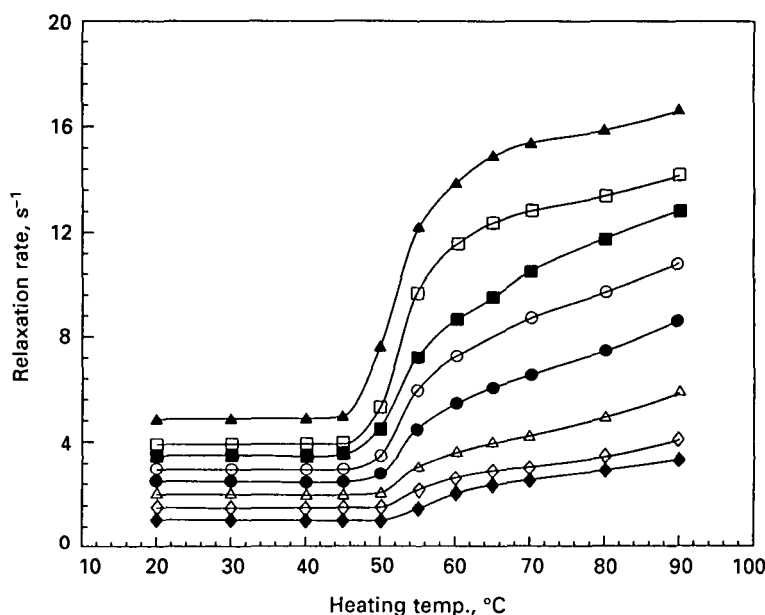


Fig. 5. Water proton T_2 relaxation rates in bovine serum albumin solution (pH 7.0) heated at various temperatures for 30 min. Protein concentrations: \blacklozenge , 3.8%; \diamond , 5.6%; \triangle , 7.5%; \bullet , 9.4%; \circ , 11.3%; \blacksquare , 13.2%; \square , 15.0%; \blacktriangle , 16.9%.

the protein content, was observed after 30 min heating. Successive treatment at temperatures between 60 and 90 °C further enhanced T_2 proton relaxation rates, but only slightly (Fig. 5).

IgG-based samples

The T_2 water proton relaxation rates were measured in 6 and 18% IgG solutions as a function of the heating temperature (Fig. 6). For both concentrations, the curves obtained were similar to those recorded with the WPC samples: no change in relaxation parameters was observed following heating up to 60 °C, sharp increases proportional to the protein content were recorded between 60 and 75 °C, and no modification was observed after further heating to 90 °C.

DISCUSSION

T_2 relaxation of the non-equilibrium water proton magnetization in whey protein aqueous solution is purely exponential. This has been already reported for most of the protein–water systems studied (Lambelet *et al.* 1988; Maquet *et al.* 1984; Oakes, 1976*a, b*; Goldsmith & Toledo, 1985; Lelièvre & Creamer, 1978; Rydzy & Skrzyński, 1980, 1982; Blicharska & Rydzy, 1979; Mahdi, 1980; Pumpernik *et al.* 1975; Richardson *et al.* 1986) and has been interpreted (Oakes, 1976*a, b*; Lelièvre & Creamer, 1978; Mahdi, 1980; Richardson *et al.* 1986) in terms of a two-state model with rapid exchange occurring between bulk water molecules and those bound to proteins. According to this model, transverse relaxation rate, T_2^{-1} , is given by:

$$T_2^{-1} = hc/(1 - hc) (T_{2b} + \tau_b)^{-1} + T_{2f}^{-1}$$

where c is the protein concentration (g/g), h is the amount of water hydrated by unit mass of protein, τ_b is the life time of a water molecule in the bound phase and

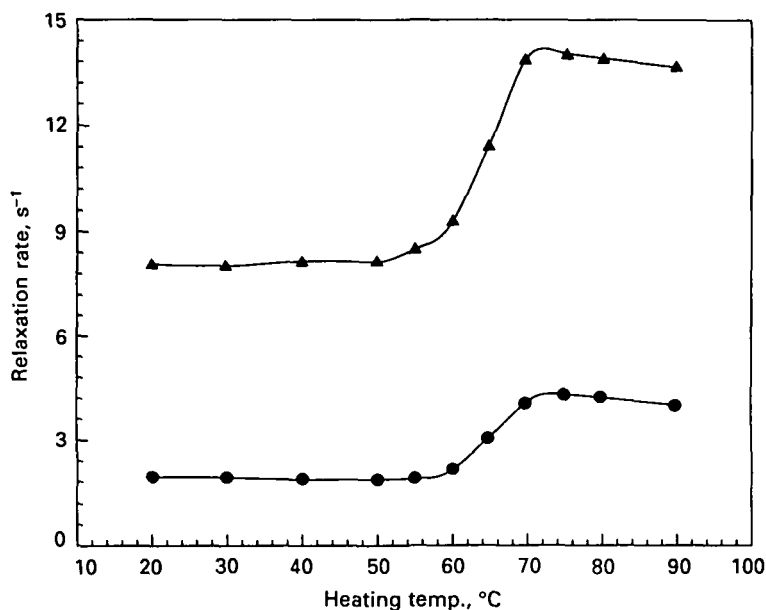


Fig. 6. Water proton T_2 relaxation rates in immunoglobulin G solution (pH 7.0) heated at various temperatures for 30 min. Protein concentrations: ●, 6.1%; ▲, 18.5%.

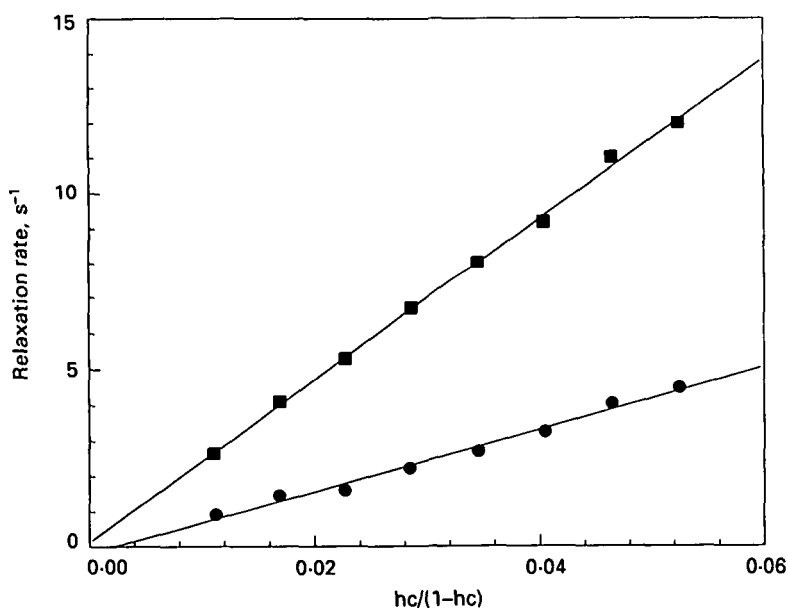


Fig. 7. Water proton T_2 relaxation rates in an industrial whey protein concentrate solution (pH 7.0) in function of $hc/(1-hc)$; see text. ●, Untreated samples; ■, samples heated at 90 °C for 30 min.

subscripts b and f refer to the bound and free states respectively. Values of h have been measured in WPCI aqueous solutions by determining the amount of unfreezable water at -35 °C using NMR spectroscopy. Within the protein concentration range 4–15%, the amount of hydrated water equalled 28%. This h value is in good agreement with corresponding data measured in whey protein solutions (Kuntz & Kauzmann, 1974).

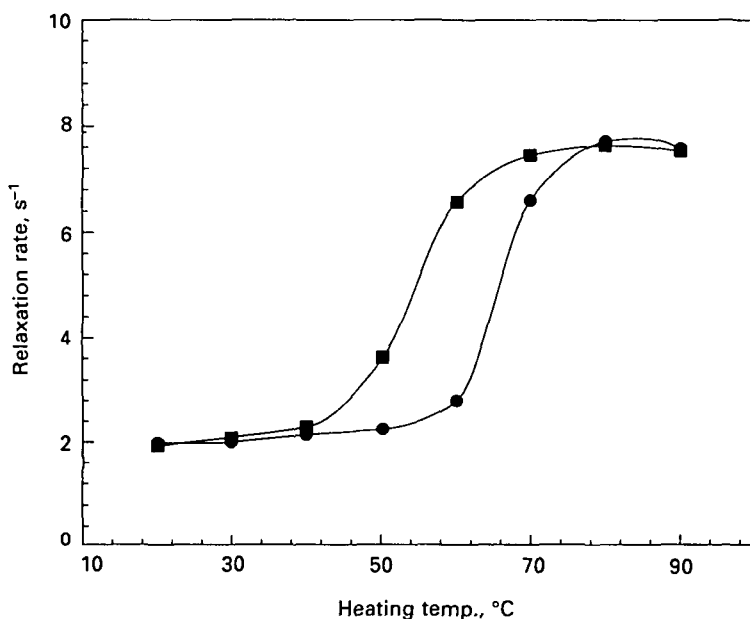


Fig. 8. Influence of 2-mercaptoethanol on the water proton T_2 relaxation rate in 10% β -lactoglobulin solution (pH 7.0) heated at various temperatures for 30 min. ●, Without and ■, with 0.3% (w/v) 2-mercaptoethanol.

The interpretation of NMR data in terms of the two-state model is confirmed by the linearity of the graph of water T_2 relaxation rates observed in WPCI aqueous solutions against $hc(1 - hc)$ (Fig. 7, lower line). Intercept of this plot approximately equals value of T_2^{-1} for pure water. Assuming $\tau_b < T_{2b}$ (Oakes, 1976a), the value of the relaxation time associated with bound water, T_{2b} , can be calculated from the slope of the graph reported in Fig. 7 (lower line). This value, 11 ms, is in good agreement with the reported value, 9.2 ms measured at 60 MHz (Oakes, 1976a). Thus, water relaxation rates observed in whey protein aqueous solutions can be understood in terms of a rapid exchange between most of the water molecules having a mobility equal to that of pure water and a small fraction of water molecules tightly bound to polar groups on the surface of the protein, water which has a mobility about 100-fold lower.

Similarly to what is recorded in untreated material, T_2 water relaxation in heat treated whey protein samples is a single experimental process, and the observed relaxation rates are linearly related to $hc(1 - hc)$ (Fig. 7, upper curve). Although a contribution to the observed water proton relaxation rates in thermally treated whey protein solutions is coming from exchange between labile protein protons and water protons (Oakes, 1976b; Lambelet *et al.* 1988), the relaxation time of bound water (T_{2b}) can be calculated from the gradient of the graph reported in Fig. 7 (upper line). Assuming $\tau_b < T_{2b}$ (Oakes, 1976b) T_{2b} equals 4 ms, e.g. a lower value than that for water bound to native whey protein (11 ms). The increase of T_2 relaxation rate during thermal treatment of whey protein aqueous solutions is therefore due to a decrease of mobility of the water fraction bound to the protein.

It is well documented that thermal treatment of whey proteins near 80 °C causes protein unfolding (Privalov & Khechinashvili, 1974; Rüegg *et al.* 1977b). The unfolding step is usually followed by protein aggregation and eventually gelation. It is not possible to attribute definitively the increase in T_2 relaxation during thermal

treatment of whey proteins to either unfolding or aggregation or both. However, polymerization through the establishment of intermolecular disulphide bonds, which takes place during thermal treatment of β -lg (Sawyer, 1968; Watanabe & Klostermeyer, 1976), is not responsible for change in T_2 relaxation rate. Indeed, as shown in Fig. 8, changes in T_2 relaxation rate during treatment of β -lg solution is also observed (although at a lower temperature) when the solution contains an excess of 2-mercaptoethanol, a product which blocks the -SH groups of the protein. In the same way, no variation in T_2 relaxation rate was observed during thermal treatment up to 95 °C of a 10% (w/v) aqueous solution of sodium caseinate, which is known not to change its structure upon heating (data not shown). Variations in relaxation parameters are certainly not related to gelation since these changes are also observed when this phenomenon does not occur, e.g. in samples with low protein concentrations.

On the other hand, it is worthwhile comparing NMR data with data obtained by differential scanning calorimetry (DSC). Indeed, thermal phenomena which are encountered during heating protein solutions (Privalov, 1974; Privalov & Khechinashvili, 1974) are not due to physical aggregation processes (Donovan & Ross, 1973) but rather to conformational changes of the protein (Privalov & Khechinashvili, 1974; Rüegg *et al.* 1977*a*; Brandts, 1969). For example, heat transition around 70 °C evidenced by calorimetry during heating of aqueous solution of β -lg (Itoh *et al.* 1976; De Wit & Swinkels, 1980; De Wit & Klarenbeek, 1981; De Wit, 1981; De Wit *et al.* 1983) occurs in the same temperature range as do conformational changes of β -lg detected by either optical rotation measurements (Dupont, 1965) or fluorescence spectroscopy (Mills, 1976). Now, this heat effect is in accordance with the change in T_2 proton relaxation rate observed in β -lg aqueous solution following 30 min heating at temperatures between 60 and 70 °C (Fig. 2). Moreover T_2 relaxation rates in β -lg solutions depend on the pH of the sample over the range 3.5–8.0 (Fig. 3), as do DSC thermograms (Itoh *et al.* 1976; De Wit & Klarenbeek, 1981). In both experiments the lowest transition is observed for the sample at pH 8.0, the highest for the sample at pH 3.5, whilst the two observed in samples at pH 6.0 and pH 7.0 are close to each other. Similarly, T_2 relaxation rate transitions in WPCI and IgG solutions are close to those observed in β -lg samples (Figs 1, 2, 6) as are close transitions observed in DSC curves of these three proteins (De Wit, 1981; De Wit *et al.* 1983). In the same way the transitions in T_2 relaxation rates observed in defatted BSA solutions heated between 50 and 60 °C are well related to corresponding DSC curves, showing an endothermic phenomenon around 65 °C (Itoh *et al.* 1976; Gumpen *et al.* 1979; De Wit *et al.* 1983). DSC curves of solutions of α -la show an endothermic phenomenon over the temperature range 50–70 °C (De Wit *et al.* 1983; Chaplin & Lyster, 1986; Pfeil, 1981; Rüegg *et al.* 1977*a*). This phenomenon has however been shown to be almost completely (around 90%) reversible on cooling (De Wit *et al.* 1983; Chaplin & Lyster, 1986; Rüegg *et al.* 1977*b*). DSC experiments have shown on the other hand that heating the α -la solutions to a higher temperature (e.g. 95 °C) and keeping them at this temperature for a short period of time (e.g. 30 min) lead to irreversible transformation of the protein (Chaplin & Lyster, 1986). Present results (Fig. 3) relating a variation in proton T_2 relaxation rates in α -la solutions heated over the temperature range 75–95 °C are therefore in fair agreement with DSC results. Obviously, the NMR method as described in the present study, e.g. with recording relaxation parameters at 20 °C, is sensitive only to irreversible transformation.

Variations in proton relaxation parameters occurring during heating of whey

Table 1. Comparison between NMR and Rowland* methods for the determination of the extent of protein denaturation in a 10 % undenatured whey protein concentrate solution (pH 7.0) heated at various temperatures for 30 min.

Heating temp., °C	Protein denaturation %	
	NMR	Rowland
60	11.3	11.0
65	55.5	45.2
70	95.3	80.0
80	100.8	80.0
90	100.0	83.1

* Rowland (1938)

protein solutions are therefore associated with protein unfolding denaturation and can be used as a probe for qualitatively investigating this transformation. The question now arises as to whether the proton relaxation parameters can be used for quantitative determination of protein denaturation or, more precisely, if the amplitude of the deviation of relaxation rate observed is somehow related to the extent of protein denaturation. In the NMR experiment, the relative amount of thermally denatured protein following heating at temperature T can, *a priori*, be expressed using the formula:

$$\% \text{ Denaturation} = \frac{R_T - R_{\min}}{R_{\max} - R_{\min}} \cdot 100$$

where R_T = Relaxation rate in sample heated at temperature T

R_{\min} = Relaxation rate in untreated sample

R_{\max} = Relaxation rate in completely denatured sample.

The validity of the NMR method for determining protein denaturation quantitatively has been tested with the laboratory prepared native whey protein concentrate sample, WPCL. For this purpose 10% WPCL solutions (pH 7.0) have been heated at various temperatures between 60 and 90 °C, held 30 min at these temperatures and then rapidly cooled to 0 °C. The degree of protein denaturation resulting from each thermal treatment was conjointly determined using the relaxation parameters and the standard Rowland method (Rowland, 1938). The two sets of data are compared in Table 1. For all heating temperatures the Rowland values are lower than the NMR values. The difference between a pair of values is in fact proportional to the extent of denaturation. The arbitrary 100 % value attributed by the NMR method to the whey heated at 90 °C is supported by the fact that no further increase in T_2 relaxation rate was observed during heating to 110 °C. Moreover the percent of denaturation measured by the standard method in a WPC sample heated at 110 °C for 30 min was still below 85 %. Such a difference between Rowland and NMR data might come from the Rowland method measuring precipitation resulting from unfolding and aggregation, whilst the NMR method is principally sensible to unfolding and not to aggregation.

Present results clearly show that whey protein irreversible thermal denaturation can be investigated using low resolution NMR spectroscopy by measuring at 20 °C T_2 water proton relaxation rate following thermal treatment. Indeed, changes in T_2 relaxation rates observed in samples with protein concentration greater than 3 % occur at denaturing temperatures, and the amplitudes of these changes can be

related to the degree of denaturation. Consequently the NMR technique might be an alternative method for measuring whey protein denaturation. In further investigations, we will examine the possibility of employing the NMR technique for studying the denaturation of proteins capable of complexing paramagnetic ions, following thermal processing up to 160 °C (De Wit & Klarenbeek, 1981) or following treatment with chemical agents.

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