Low-Field Nuclear Magnetic Resonance Relaxation Study of the Thermal Denaturation of Transferrins

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The NMR method previously described by Lambelet et al. (*J. Dairy Res.* **1989**, *56*, 211–222) has been evaluated for the investigation of thermal and acid denaturation of transferrins in aqueous solution. Heating an apoovotransferrin or an apolactoferrin solution resulted in a slight increase in T_1^{-1} and an important increase in T_2^{-1} relaxation rates within the denaturation range. The same treatment applied to corresponding iron-saturated proteins resulted in a decrease in these parameters due to modifications of the metal–protein complex accompanying the denaturation. It is proposed that these changes in NMR relaxation parameters can be used to monitor thermal denaturation of transferrins. Acid treatment led to a decrease in relaxation parameters for both apoovotransferrin and iron-saturated (down to pH 4) ovotransferrin. These variations in NMR parameters were related to protein denaturation and indicated that the protein modifications due to acid treatment were different from those occurring during heating.

1. INTRODUCTION

The transferrins (Aisen and Listowsky, 1980; Baker et al., 1987) comprise three major types of proteins: lactoferrin found in milk and other secretions as well as in leucocytes; ovotransferrin from egg; and serum transferrin responsible for iron transport in the serum. The fundamental chemical and physical properties of these proteins are very similar. Each is a monomeric glycoprotein of molecular weight ca. 80 000 capable of reversibly binding two iron(III) ions, or other transition-metal ions, per molecule concomitantly with two carbonate ions. In aqueous solution transferrins, both in the apo or in the iron-saturated form, are denaturated by heat (Lyster et al., 1984), by lowering the pH (Yeh et al., 1979), or by treatment with urea (Yeh et al., 1979; Teuwissen et al., 1974).

Because of the relative ease and reliability with which paramagnetic transition-metal ions can be inserted into specific sites in the transferrins, electron spin resonance spectroscopy (ESR) has been particularly useful for the investigation of these proteins. Information on the conformation of a protein in an aqueous solution can, on the other hand, be obtained by using pulsed NMR spectroscopy by determining the relaxation times of the hydrogen atoms of the water molecules. Thermal denaturation of proteins has thus been investigated by monitoring the longitudinal (T_1) or the transverse (T_2) relaxation times during the heating (Goldsmith and Toledo, 1985; Rydzy and Skzynski, 1980; Blicharska and Rydzy, 1979; Pumpernik et al., 1975). However, since relaxation times are temperature dependent, we have developed a method for the study of protein denaturation in which the measurements are always carried out at the same temperature (Lambelet et al., 1989). A decrease in water proton T_2 relaxation time was observed after heating of diamagnetic whey protein aqueous solution. This variation in NMR relaxation parameter could be associated with protein unfolding during denaturation (Lambelet et al., 1989).

Introducing a paramagnetic ion into a sample drastically modifies the NMR relaxation parameters measured in this sample. To know whether the NMR method could be used for the study of transferrin denaturation, water proton relaxation parameters have been measured during either thermal or chemical treatment of transferrin in aqueous solutions. Changes observed in the NMR relaxation parameters are discussed in terms of modification of the paramagnetic center recorded by ESR spectroscopy.

2. MATERIALS AND METHODS

Materials. Apoovotransferrin (type I, lot 107 F 8020) and iron-saturated (type II, lot 104 F 8065) ovotransferrin were purchased from Sigma Chemical Co. (St. Louis, MO). The iron-saturated ovotransferrin was purified by dialysis at 4 °C for 45 h against water at pH 7 and subsequently lyophilized. The apoovotransferrin and iron-saturated ovotransferrin have protein contents (gas chromatography N × 6.1) of 89% and 88%, respectively.

A mixture of lactoferrin and lactoperoxidase was isolated from skim milk by absorption on Sephadex gel, elution with a salt solution, and demineralization by electrodialysis. Lactoferrin was then separated by selective absorption on Sephadex gel in a solution of controlled ionic strength. The lactoferrin was saturated with iron(III) chloride in the presence of $NaHCO_3$.

The iron-saturated lactoferrin had a protein content (gas chromatography N × 6.38) of 89%. The corresponding apoprotein was obtained by dialyzing three times against 0.1 M phosphate buffer, pH 4.6, containing 1% EDTA and then against distilled water (Spik et al., 1978). It has a protein content (gas chromatography N × 6.38) of 88%.

Sample Preparation. Ovotransferrin aqueous solutions were prepared by mixing the protein with distilled water to give 5% and 10% (w/v) protein solutions. The pH of the solutions was adjusted to 7.0 with 0.1 M NaOH for thermal denaturation studies and to levels between 2.0 and 7.0 with either 0.1 M HCl or 0.1 M NaOH for chemical denaturation studies. The samples were then degassed and introduced into an NMR sample tube, and the tube was sealed. Thermal denaturation was achieved by heating the sample for 30 min at defined temperatures between 20 and 120 °C. Denaturation was investigated after each thermal

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treatment by measuring T_1 and T_2 water proton relaxation times in samples thermostated at 20 °C for 30 min.

NMR Measurements. NMR measurements were run on a Minispec PC20 and on a Minispec PC 120 (Bruker Physik AG, Karlsruhe, Germany) at a resonance frequency of 20 MHz. T_2 relaxation times were determined from the spin echo decay curve (169 echoes) observed with a Carr-Purcell-Meiboom-Gill pulse sequence. T_1 relaxation times were determined from the curve obtained with a series of 180°–τ–90° pulse pairs for increasing pulse spacings and with recycling times (RD) between such pairs of 10 s. The T_1 or T_2 relaxation times were calculated on an Hewlett-Packard 950 computer by performing nonlinear regression of exponentials to the corresponding curves.

ESR Measurements. First-derivative ESR spectra were recorded at 77 K on Varian E-109 Century series Mark III and on Bruker 200 D spectrometers (X band) with 100-kHz magnetic field modulation. The microwave frequency was measured by using a Hewlett-Packard 5342 A frequency counter and the magnetic field with a Varian E-500-2 NMR Gaussmeter calibrated with perylene cation radical in H_2SO_4 as the g-factor reference (Wertz and Bolton, 1972). Relative intensities of ESR signals were measured by using samples crossing the cavity through and through so as to minimize the influence of the sample position on the measurements.

Light Absorption. Absorbances were measured on a Hewlett-Packard 8450 A diode array spectrophotometer.

3. RESULTS AND DISCUSSION

Water proton T_1 and T_2 relaxations in apoovotransferrin or lactoferrin could be analyzed in terms of a single exponential characterized by relaxation times T_1 or T_2, respectively. This has already been reported for most of the protein–water systems studied (Oakes, 1976a,b; Lelièvre and Creamer, 1978; Mahdi, 1979; Richardson et al., 1986; Lambelet et al., 1989; Myers-Betts and Baianu, 1990a) and is consistent with a fast exchange between bound and free water populations (Derbyshire, 1982).

Longitudinal and transverse relaxation rates (R_1 and R_2) of water protons in apoovotransferrin are linearly related to the protein concentration (Figure 1). This indicates that the protein activity is small in this concentration range and does not contribute to the ^1H relaxation rates (Kumosinski and Pessen, 1982; Myers-Betts and Baianu, 1990b).

The interpretation of ^1H NMR relaxation data can, on the other hand, be complicated by cross-relaxation between water and protons (Koenig et al., 1978; Bryant and Shirley, 1980; Kumosinski and Pessen, 1989) as well as by chemical exchange (Piculell, 1986). Cross-relaxation affects principally the longitudinal (T_1) relaxation rate. As the rate of magnetization transfer from water to protein protons is proportional to the protein concentration (Kumosinski and Pessen, 1989), significant cross-relaxation would therefore lead to an increase of the T_2/T_1 ratio as a function of the protein concentration. The decrease in the T_2/T_1 ratio observed for the apoovotransferrin aqueous solution (Figure 2) indicates that cross-relaxation does not make a major contribution to relaxation in the protein concentration range 2–10%. This is confirmed (Kumosinski and Pessen, 1982) by the longitudinal (T_1) relaxation of water protons being a single exponential in apoovotransferrin aqueous solutions. The gradual decrease in the T_2/T_1 ratio as a function of the protein concentration (Figure 2) might reflect an exchange between water proton and protein proton (Myers-Betts and Baianu, 1990a). Such chemical exchange in protein–water systems has already been postulated (Oakes, 1976a; Lambelet et al., 1988; Hills et al., 1990).

T_1 and T_2 relaxations in iron-saturated transferrins solutions are also purely exponential. Corresponding relaxation rates are, however, much higher than those

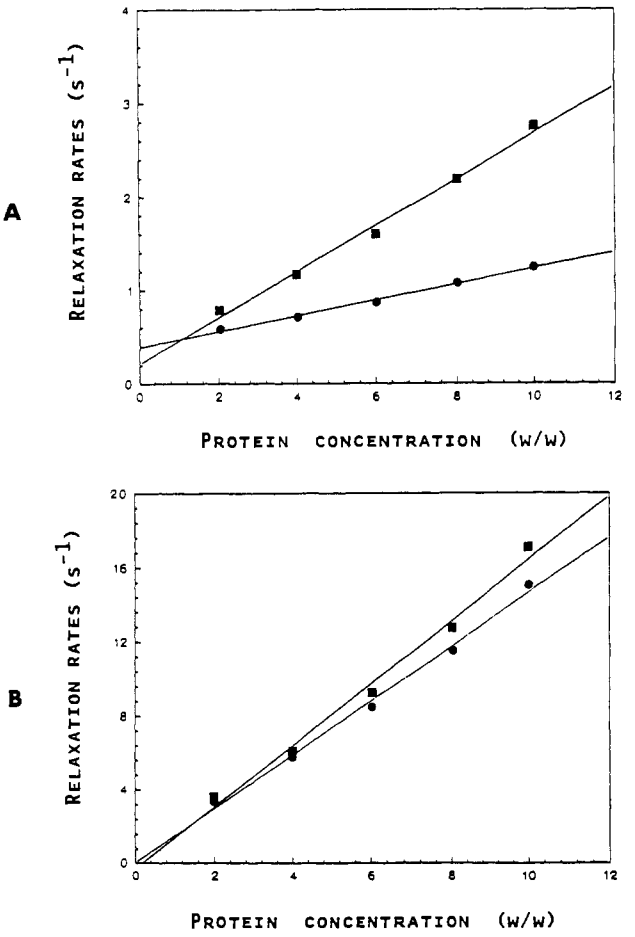

Figure 1. Water proton longitudinal and transverse relaxation rates in ovotransferrins solutions as a function of protein concentration. (A) Apoovotransferrin: (●) longitudinal relaxation rate; (■) transverse relaxation rate. (B) Iron-saturated ovotransferrin: (●) longitudinal relaxation rate; (■) transverse relaxation rate.

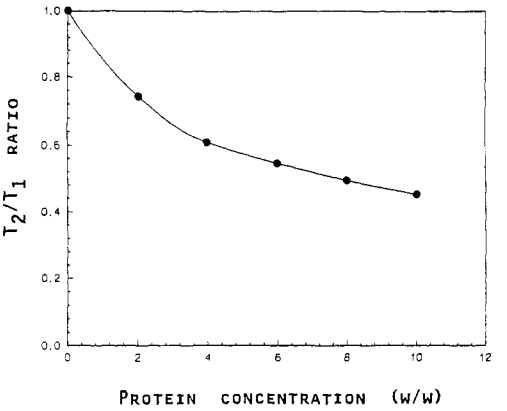

Figure 2. Variation of the T_2/T_1 ratio in apoovotransferrin solutions as a function of the protein concentration.

measured in the absence of iron. In iron-saturated ovotransferrin solution, the longitudinal and transverse relaxation rates are linearly related to the protein concentration but are not significantly different from each other (Figure 1B). This implies that relaxation in iron-saturated transferrins solutions is strongly influenced by the metal ion (Koenig and Schillinger, 1969).

Thermal Denaturation. Ovotransferrin is a thermolabile protein. Denaturation of apoovotransferrin in unbuffered water at pH 7, as monitored by differential scanning calorimetry (DSC), occurs at 60 °C (Donovan et

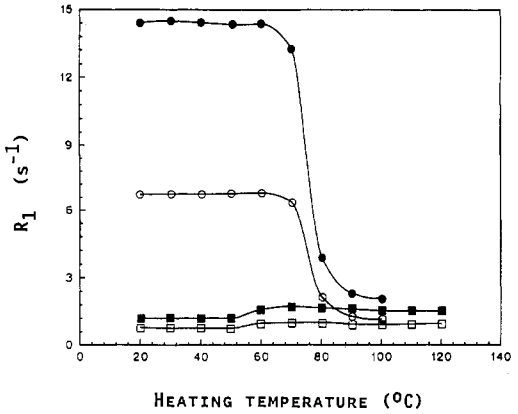

Figure 3. Water proton longitudinal relaxation rates in ovotransferrin solutions (pH 7.0) heated at various temperatures for 30 min. Apoovotransferrin 5% (□) and 10% (■) solution; iron-saturated ovotransferrin 5% (○) and 10% (●) solution.

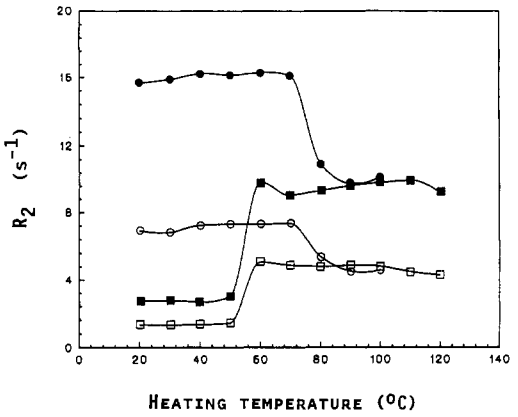

Figure 4. Water proton transverse relaxation rates in ovotransferrin solutions (pH 7.0) heated at various temperatures for 30 min. Apoovotransferrin 5% (□) and 10% (■) solution; iron-saturated ovotransferrin 5% (○) and 10% (●) solution.

al., 1975). In terms of longitudinal relaxation rate, this denaturation is expressed by the very slight increase observed between 50 and 60 °C (Figure 3). This is not surprising as similar results have been obtained by measuring longitudinal water proton relaxation rates during thermal denaturation of whey proteins (Oakes, 1976b). In fairly good agreement with DSC results (Donovan et al., 1975; Hegg et al., 1978) a significant rise in transverse water proton relaxation rates, proportional to the protein content of the sample, was observed in apoovotransferrin aqueous solutions (pH 7.0) after they were heated for 30 min at temperatures between 50 and 60 °C (Figure 4). Thermal transitions observed by DSC during heating of globular protein solutions are related to protein unfolding denaturation (Donovan and Ross, 1973; Privalov and Khechinashvili, 1974; Brandts, 1969). The variation in transverse relaxation rates observed during heating conalbumin solution can therefore be associated with changes in the conformation of the protein. An increase in transverse relaxation rates has already been observed following thermal treatments of various proteins such as whey and egg white proteins (Oakes, 1976b; Lambelet et al., 1988, 1989). In the case of bovine serum albumin (BSA), it was shown that on thermal denaturation the protein proton transverse relaxation rates become much higher, but the longitudinal relaxation rates remain unchanged (Oakes, 1976b). This indicates that the protein chain becomes much less mobile. The increase in the T_2 relaxation rate of water proton during thermal denaturation of BSA was thus explained in terms of enhancement

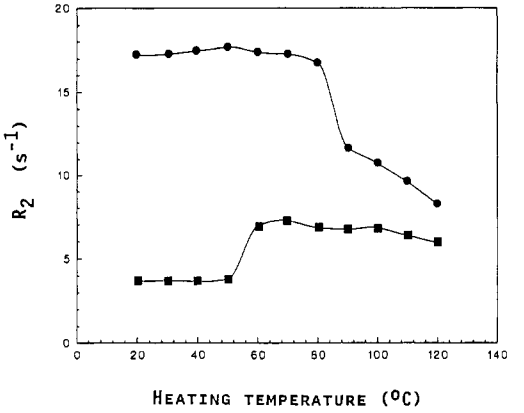

Figure 5. Water proton transverse relaxation rates in 10% lactoferrin solutions (pH 7.0) heated at various temperatures for 30 min. (■) Apolactoferrin; (●) iron-saturated lactoferrin.

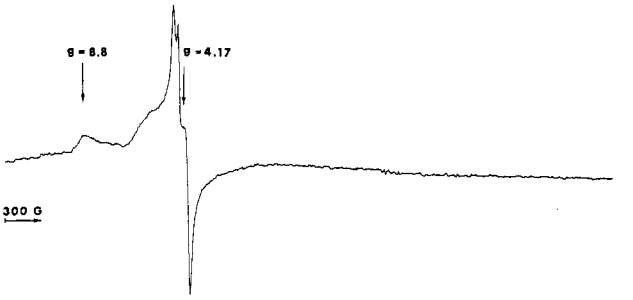

Figure 6. ESR spectrum of native iron-saturated ovotransferrin solution (10%) frozen to 77 K.

in the bound water transverse relaxation rate associated with a decrease in protein chain mobility. The similar change in water proton relaxation rate observed during heating of conalbumin solution is to be interpreted in the same way. It is worthwhile mentioning, however, that the native structure of BSA is not extensively disrupted on thermal denaturation (Oakes, 1976b) and that a decrease in T_2 relaxation rate of water protons during thermal treatment of proteins may also be encountered, as, for example, with corn zein (Myers-Betts and Bainau, 1990a).

Lactoferrin is also thermosensitive. In its apo form lactoferrin dissolved in simulated milk ultrafiltrate (SMUF) denatures at ca. 65 °C as shown by DSC calorimetry (Rüegg et al., 1977). The increase in water proton transverse relaxation rates following heating of apolactoferrin aqueous solutions in the temperature range 50–60 °C (Figure 5) can therefore be related to unfolding denaturation.

Binding of iron to ovotransferrin markedly stabilizes this protein against heat denaturation (Azary and Feeney, 1958). Thus, the denaturation temperature of iron-saturated ovotransferrin in Tris buffer (pH 7.5) as determined by DSC was found at 84 °C, a value nearly 20 °C higher than that determined for the apoovotransferrin (Donovan and Ross, 1975). Accordingly, a substantial decrease either in longitudinal (Figure 3) or in transverse (Figure 4) water proton relaxation rates is observed between 70 and 90 °C. This modification of NMR relaxation parameters can be understood by examination of the ESR spectra. The ESR spectra of the two iron-saturated transferrin (ovotransferrin and lactoferrin) solutions frozen to 77 K were similar (see, for example, the ovotransferrin spectrum depicted in Figure 6). As previously reported (Aasa, 1972; Aisen et al., 1974) these spectra correspond to high-spin iron(III) ions (rhombic symmetry) and are characterized by a weak signal at g = 8.8 and a cluster A of three narrow lines near g = 4.2; a

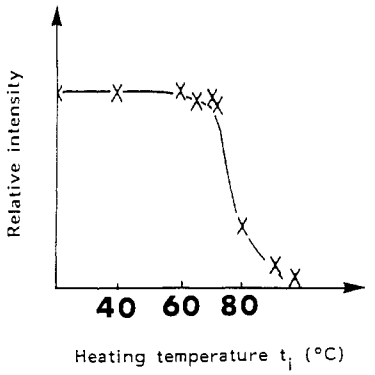

Figure 7. Relative intensity (in arbitrary units) of ESR signal A in ovotransferrin solutions heated at various temperatures t_i for 30 min.

broad signal is also observed near these latter lines. To determine if an increase of the temperature has an effect on the amount of iron(III) ions bound to the proteins, solutions of iron-saturated lacto- and ovotransferrins were heated for 0.5 h at a fixed temperature t_i before the ESR spectra were recorded. Both transferrins gave similar results.

Figure 7 clearly shows that thermal treatment up to 70 °C does not have any effect on the amount of iron(III) ions bound to ovotransferrin; a drastic decrease in the intensity of the ESR signal appears between 70 and 90 °C. This variation parallels the temperature dependence of the NMR relaxation rates (Figures 3 and 4). Above 90 °C the original iron(III)–protein complex almost completely disappeared for both ovotransferrin and lactoferrin. Thus, in untreated samples, water proton relaxations are governed by the magnetic interaction between water proton molecules tightly bound to the protein and the metal ion in the high-spin state, the latter being buried only 10 Å beneath the protein surface (Brock, 1985). Accordingly, high relaxation rates are registered for the water protons. Since the complex between iron and ovotransferrin is destroyed during thermal treatment, the iron is most certainly removed from the protein. Iron(III) completely hydrolyzes under the experimental conditions to form ferric hydroxide complexes (Cotton and Wilkinson, 1967) which do not affect the proton relaxation of the bulk water (Aisen et al., 1966). Thus, low relaxation rates, in the same order of magnitude as those measured in heated apoovotransferrin, are observed. Instead of the expected increase associated with protein unfolding, a decrease in relaxation rates is observed during iron-saturated ovotransferrin denaturation due to the concomitant release of the metal ion. Denaturation of iron-saturated lactoferrin in SMUF has been shown by DSC to occur around 84 °C (Rüegg et al., 1977). A reduction of transverse relaxation rates following the heating of iron-saturated lactoferrin in aqueous solution is observed in the temperature range 70–90 °C. As for ovotransferrin, this variation in NMR relaxation parameters is due to the dissociation of the metal–protein complex and is associated with protein denaturation.

Acid Denaturation. The isoelectric point of ovotransferrin is slightly below pH 7.0 (Warner and Weber, 1951). Extreme acidic conditions will lead to a change in the charge relationship on the ovotransferrin system and consequently to its denaturation. Water proton longitudinal and transverse relaxation rates measured in apoovotransferrin indicate, however, that the nature of acid denaturation is different from that of thermal denaturation. Whereas thermal unfolding denaturation of apoovotransferrin results in an increase in the transverse relaxation rate and does not significantly modify the longitudinal relaxation rate (Figures 3 and 4), acid denaturation decreases these NMR parameters (Figure 8). The decrease in longitudinal or transverse relaxation rates during acidic treatment of apoovotransferrin solutions is nearly linear in the pH range 2–7. This variation in NMR parameters is certainly related to modification of the protein structure as neither the longitudinal nor transverse relaxation rate of water protons is reported to show pH dependency (Glick and Tewari, 1966).

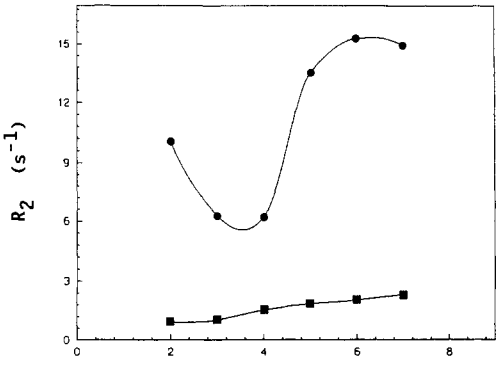

Figure 8. Variation of water proton transverse relaxation rates with pH of ovotransferrin solutions. (■) 10% apoovotransferrin solution; (●) 10% iron-saturated ovotransferrin solution.

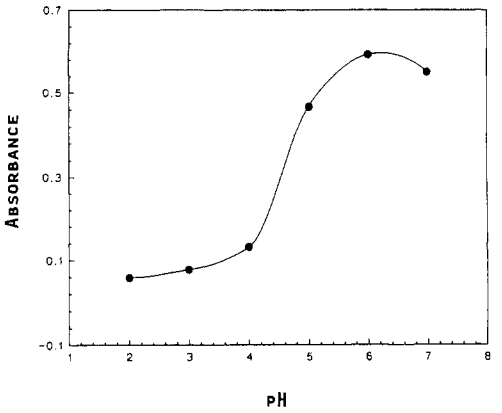

Figure 9. Variation with pH of the light absorption at 458 nm of iron-saturated ovotransferrin solutions.

For iron-saturated ovotransferrin, denaturation by acid is also different from thermal denaturation. Indeed, contrary to what is observed in the case of thermal denaturation, the relaxation rates measured in iron-saturated ovotransferrin solutions in the pH range 2–7 are always higher than corresponding relaxation parameters measured in apoovotransferrin. Whatever the pH of the solution between 2 and 7, the metal ion influences the water proton relaxation. As previously reported (Yeh et al., 1979), the light absorption around 460 nm in iron-saturated ovotransferrin solution decreases if the pH is lowered (Figure 9). Within the pH range 3–4 to 7, this change in visible light absorption is on a par with the variation of longitudinal or transverse relaxation rates recorded in the same solutions (Figures 8 and 9) (Aisen et al., 1966). This indicates that the structure of the metal–protein complex is modified during acid denaturation and that this modification can be followed by NMR relaxation measurements.

Below pH 3 there is, however, a discrepancy between visible light absorption and longitudinal or transverse relaxation rates. While the absorption coefficient further decreases from pH 3 to pH 2, the longitudinal as well as

transverse relaxation rates increase under the same conditions. This might originate from the iron-saturated ovotransferrin denaturation being reversible down to pH 3 only (Yeh et al., 1979). Under the present conditions (pH 2, ionic strength not controlled), the acid denaturation of iron-saturated ovotransferrin is not completely reversible, as shown by the lower light absorption and relaxation rates in the renatured as compared to the native ovotransferrin.

4. CONCLUSION

Water proton longitudinal and transverse relaxation rates are modified during pH or heat-induced denaturation of ovotransferrin in either its apo or iron-saturated form. The variation of these NMR parameters during acid or thermal denaturation of ovotransferrin solutions is, however, different, indicating that these denaturations produce modifications of the protein which are different in nature.

Thermal denaturation of ovotransferrin induces modification of the protein structure that can be followed by using the NMR relaxation probes. As for milk proteins, water proton transverse relaxation rates in apoovotransferrin solution increase as the protein undergoes thermal unfolding denaturation. In iron-saturated ovotransferrin, thermal denaturation is associated with a permanent modification of the metal–protein complex, resulting in a decrease in longitudinal and transverse water proton relaxation rates during heating. These changes in NMR relaxation rates observed after heating of both apoovotransferrin and iron-saturated ovotransferrin solutions occur in the same temperature range as do heat transitions recorded by calorimetry: i.e., between 50 and 60 °C and between 70 and 80 °C for the apoovotransferrin and iron-saturated ovotransferrin, respectively (Donovan and Ross, 1975). Relaxation rates, and in particular the transverse relaxation rate, are thus a good probe for the qualitative study of the thermal denaturation of ovotransferrin. The amplitude of the variations in NMR relaxation parameters is, on the other hand, a function of the amount of ovotransferrin undergoing denaturation. Consequently, NMR relaxation parameters and particularly the transverse relaxation rate should be useful for the quantitative determination of denaturation in ovotransferrin. The NMR method could also be applied to the study of thermal denaturation of the other transferrins; indeed, substantially similar variations in NMR longitudinal and transverse relaxation rates were observed during the thermal treatment of apolactoferrin and iron-saturated lactoferrin solutions.

The decrease of the water proton relaxation rates during acid treatment (down to pH 4) of iron-saturated ovotransferrin parallels changes in the metal–protein complex and can therefore serve as a basis for studying acid denaturation. Similarly, the continuous decrease observed in the NMR parameters during acid treatment of apoovotransferrin can be assumed to be related to protein denaturation.

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