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Tyrosine group behaviour in bovine α-lactalbumin as revealed by its Raman effect

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Abstract. Side group behaviour is often used for conformational studies of proteins. We have performed Raman spectroscopic measurements on the tyrosine groups of bovine α-lactalbumin. The 850/830 cm^{-1} doublet intensity ratio is a direct measure of the negative charge state of the phenolic oxygen and of the tyrosine environment. pH measurements confirm the existence of an acid conformer of BLA, that is comparable to, but clearly distinguishable from the apo-conformer. Following the Siamwiza theory, the Tyr groups in this partially unfolded state are situated in a more hydrophobic environment. Observation of Tyr groups behaviour in the denaturated states obtained by thermal or chemical treatment leads us to the same conclusion. However, the behaviour of tryptophan groups is quite different. In an unfolded state, the Trp residues are mostly exposed to the solvent.

The stabilizing role of Ca^{2+} and Na^{+} ions in BLA is also investigated.

Key words: Raman spectroscopy, α-lactalbumin, tyrosine, protein conformation

Introduction

Bovine α-lactalbumin (BLA) is a small protein (14.4 kilodalton) containing four each of tryptophan, tyrosine and phenylalanine side groups. Since Hiraoka et al. (1980) first showed that BLA was a calcium metalloprotein, tryptophan fluorescence (Permyakov et al. 1981; Hanssens et al. 1984; Permyakov et al. 1985), ESR (Murakami et al. 1982) and NMR (Gerken 1984) experiments have confirmed that BLA contains a high affinity binding site for divalent cations. The binding of Ca^{2+} ions, in the absence of other ions such as Na^{+} causes a conformational change which restores the native tertiary structure and results in a transfer of some exposed tryptophan residues from the protein surface to the rigid non-polar interior. This conformational change, reflected in a decrease in the quantum yield and in an spectral shift towards shorter wavelengths resembles the phenomena observed during the transition from the acid (U) to the native (N) state of the protein. Because of the competition between protons and Ca^{2+} ions for the same binding site, the acid form of BLA resembles the demetallized apo-form of the protein. In both these partially unfolded states, the tryptophan residues are exposed to a more polar environment. Heating or addition of increasing concentrations of denaturant induces similar partially unfolded states, although small differences in their tryptophan environment properties can be observed (Permyakov et al. 1985).

In this kind of investigation, the experimental conditions must be strictly controlled in order to allow interpretion of the data in an unambiguous way. It has been recently shown (Permyakow et al. 1985; Hiraoka and Sugai 1985) that addition of NaCl to the apo-conformer in concentrations up to 0.1 M has the same effect as the binding of one Ca^{2+}. The Na^{+} ion binds to the Ca^{2+} binding site with an apparent binding constant $K_{app} = 10^2\ M^{-1}$ while K_{app} for Ca^{2+} is of the order of $10^8\ M^{-1}$. Kronman and Bratcher (1982) have reported on the influence of even micromolar concentrations of metal chelating buffers such as EDTA and EGTA on the protein conformation. Recently however, it has been shown (Mitani et al. 1986), that these chelators have no significant effect on the thermal unfolding curves of the apo-protein. *Tris* buffer at 40 mM has little effect on the tertiary structure of the apo-conformer but higher concentrations can induce conformational transitions (Hiraoka and Sugai 1985).

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Abbreviations: BLA, bovine α-lactalbumin; EGTA, ethylene glycol bis (β-aminoethyl ether) N,N′-tetraacetic acid; Tyr, tyrosine; *Tris*, *tris*(hydroxymethyl)aminomethane

In this paper we show that Raman spectroscopy can contribute considerably to the understanding of conformational changes in proteins. In particular the intensity ratio of the tyrosine doublet, $I_{850/830}$, can give information about the binding state of these residues and about their location in the protein. Most of the conclusions of tryptophan fluorescence studies are confirmed by our study of the spectrum of the tyrosine groups of BLA. We find that the binding of Ca^{2+} to BLA stabilizes the protein and that removal of Ca^{2+} induces conformational changes similar to those found in the $N \rightarrow U$ transition. This "looser" form of BLA is also found upon heating and upon adding guanidine hydrochloride. The most important result however concerns the location of the tyrosine residues in the protein. In the tryptophan experiments, the partial unfolding of BLA involves an increase in the number of tryptophans exposed to the solvent. In contrast, our results indicate that, during the unfolding process, the tyrosine residues are directed to the hydrophobic interior of the molecule and are less exposed to the solvent.

Materials and methods

The lyophilized BLA powder was purchased from Sigma Chemicals Co. Apo-α lactalbumin was prepared as described by Van Ceunebroeck et al. (1985). The protein concentrations were determined with a Beckman D spectrophotometer at 280 nm using a value $E_{1\%} = 20.1$. The $CaCl_2$ used to calcify the Sigma product is an ultrapure Merck product. The Ca^{2+} and Na^+ contents of the samples were determined by atomic absorption. All protein solutions (10 to 20 weight %) were introduced into a Kimax capillary and were shaken to bring the sample droplet down to the closed end of the capillary. Raman spectra were recorded on a Coderg spectrometer equipped with a CR3 argon ion laser. The 514 nm laser excitation line was used with a maximum power of 200 mW at the sample. The scattered light is collected in a double monochromator with cooled photomultiplier (RCA C31034A) and treated with photon counting methods. All data were stored in an Apple II computer and were seven-point smoothed (Savitsky and Golay 1964). Peak heights were determined with reference to a computer chosen linear baseline in this spectral region.

Results

1. Interpretation of the tyrosyl peaks in the Raman spectrum

BLA contains four tyrosine residues at position 18, 36, 50 and 103 (Brew et al. 1967). The global contributions of these four Tyr groups are observed at different positions in the Raman spectrum. As in all Tyr containing proteins, peaks around 650(m), 820(s), 852(s), 1,180(m), 1,210(s) and 1,621(w) cm^{-1} (Tsuboi, 1976) are observed in BLA (Fig. 1). As phenylalanine also contributes to the intensity of the last three peaks, they cannot be used to study the Tyr environment. Most attention therefore is given to the doublet in the 800 cm^{-1} region.

According to the investigations of Siamwiza et al. (1975), this doublet is due to Fermi-resonance between the ring-breathing fundamental vibration and the overtone of an out-of-plane ring-bending vibra-

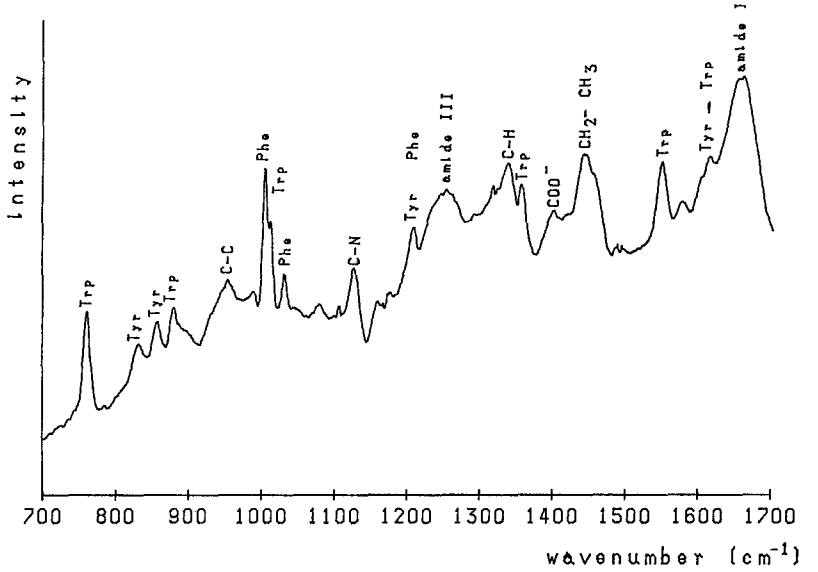

Fig. 1. Raman spectrum (700–1,700 cm^{-1}) of BLA in distilled H_2O. Experimental conditions: laser power 200 mW, scan speed 1 cm^{-1}/s, slit width 500 μm, number of accumulated spectra 40

tion of the parasubstituted benzenes. The intensity ratio of the two components, $R = I_{850}/I_{830}$, is found to be sensitive to the nature of the hydrogen bonding of the phenolic hydroxyl group or its ionization state and much less to the change in the environment of the benzene ring or the conformation of the backbone. The ionization of the hydroxyl group on *p*-cresol is closely related to the reversal of the relative intensities of the Raman doublet: below the pK_A (= 10.5) a high ratio is found, above pH = 10.5 the ratio becomes smaller than 1. The overall effect of hydrogen bonding can be summarized as follows: as the phenolic oxygen acts as a proton donor to a negatively charged acceptor, the ratio R has a low value (mostly < 0.9). If on the other hand, the phenolic oxygen acts as a much weaker proton donor or as an acceptor to an external acidic proton, $R > 0.9$. The tyrosine doublet intensity is thus directly affected by the partial negative charge on the phenolic oxygen. This conclusion was drawn from a great number of experiments on *p*-cresol and other model compounds with para-disubstituted benzenes at various pH values and in different solvents. A reanalysis of this data by McHale (1982) showed that the uncoupled intensity of the overtone mode, that is assumed to be zero in the Siamwiza theory, is not always negligible, but the fundamental interpretation of the tyrosine doublet is maintained. When this theory is applied to proteins (Siamwiza et al. 1975), two cases have to be considered: for a tyrosine, exposed on the surface of a protein in aqueous solutions, the state of the phenolic OH is that of a simultaneous acceptor and donor of moderate to weak bonds and therefore the doublet intensity ratio will fall in the range 1.45 to 0.9. For a tyrosine buried within the protein in hydrophobic regions the ratio should vary from 1.45 to 0.3 in the case of extreme H-bonding to a negative acceptor.

In this way, Craig and Gaber (1977) showed that in human carbonic anhydrase B, 3.7 Tyr residues are buried and 4.3 are exposed to the solvent. Applying the same reasoning, Chen and Lord (1980) showed that in α-chymotrypsin all four Tyr are weakly hydrogen bonded, while in trypsin with ten Tyr most of the Tyr are accessible to solvent molecules. In their study of the thermal unfolding of ribonuclease A, Chen and Lord (1976) measured a continuous and gradual transition curve where the native intensity ratio 8/10 at 40 °C is converted to 10/6 at 70 °C.

2. *Apo-α-lactalbumin*

The thermal unfolding of apo-BLA was investigated at pH = 8 in 40 m*M* *Tris* and 5 m*M* Na^+. At 6 °C $R = 1.3$, at 27.5 °C $R = 1.1$ and at 46.5 °C $R = 0.7$. The ratio R thus decreases at higher temperatures.

Permyakov et al. (1985) found from tryptophan fluorescence measurements at pH = 8 that the midpoint of the thermal transition for apo-BLA lies at 31 ± 1 °C and that the complete transition to the high temperature conformer is achieved near 45 °C. They also showed that this midpoint shifts to higher temperatures upon Ca^{2+} and Na^+ binding. These results are also confirmed by recent circular dichroism measurements (Kuwajima et al. 1986). They found that at pH = 7 the Ca^{2+} free BLA in $Na^+ = 0.1$ *M* was folded below 20 °C and that the unfolding process is achieved at 55 °C. Our results indicate that in the thermal unfolding process the tyrosine environment is clearly affected. Following the Siamwiza theory the rather high R value at 6 °C reflects the formation of hydrogen bonds of moderate strength by the phenolic OH to water molecules or the formation of a hydrogen bond between the hydroxyl oxygen of tyrosine and an external hydrogen, for instance from a COOH group. Since at pH = 8 the COOH groups are dissociated, the latter possibility can be excluded. Furthermore, no hydrogen bond from the tyrosyl group to the negative acceptor group COO^- occurs, because in that case, according to the Siamwiza theory, a low intensity ratio would be expected. We propose therefore that in the folded state of apo-α-lactalbumin, found at low temperature, most of the four Tyr groups are exposed to water.

At higher temperatures the apo-conformer unfolds and the R value decreases to 0.7 at 46.5 °C. Following the Siamwiza theory such a low R-value for a Tyr group in a protein suggests that it is buried in a hydrophobic region within the protein where H-bonding occurs to a negative acceptor. In their NMR experiments, Kuwajima et al. (1986) observed a one-proton doublet in the spectra taken at 40° and 50 °C. For a tyrosyl ring such an observation requires that the ring is immobilized inside the protein molecule. The fact however that this resonance doesn't undergo line broadening at higher temperatures led the authors to assign this resonance to a proton of a Trp residue, rather than to an immobilized tyrosyl group.

3. *Effects of Ca^{2+} and Na^+ ions*

Ca^{2+} ions have a considerable effect on the stability of BLA (Hiraoka et al. 1980; Hanssens et al. 1984; Permyakov et al. 1985). It is generally accepted that BLA can tightly bind one Ca^{2+} and that thermal unfolding is retarded by the presence of Ca^{2+} ions. Thermal transition curves based on fluorescence

polarization data (Permyakov et al. 1985) and on circular dichroism and NMR data (Kuwajima et al. 1986) show a net shift to higher temperatures for BLA with increasing Ca^{2+} concentration. One should note, however, that both the Ca^{2+} bound and the Ca^{2+} free forms of BLA can assume the same folded conformation at low temperatures. Only when the tertiary structure of the protein begins to break up do the specific effects of Ca^{2+} and Na^{+} ions appear. Table 1 summarizes our measurements on Sigma BLA in 40 m*M Tris* buffer at pH = 8 in the presence of Ca^{2+} and Na^{+} ions. All these experiments are performed at $T = 25\,°C$ where the thermal transition for apo-BLA has just started.

Table 1. Influence of Ca^{2+} and Na^{+} concentration on R at pH = 8 in 40 m*M Tris* and at $T = 25\,°C$

	(BLA) [m*M*]	(Ca^{2+}) [m*M*]	(Na^{+}) [m*M*]	R
1)	5.6	1.8	0.9	1.17
2)	5.6	6.6	0.8	1.40
3)	5.3	1.8	10	1.27
4)	5.4	1.8	100	1.48

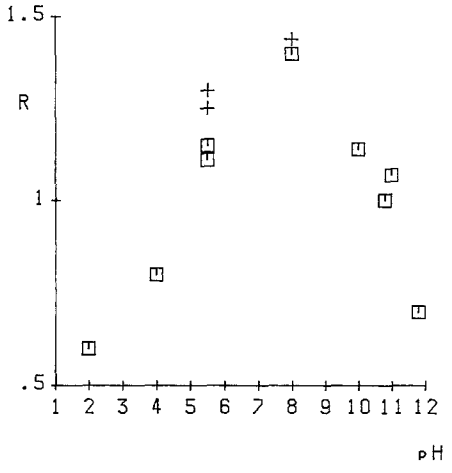

Fig. 2. pH dependence of the tyrosine doublet intensity ratio for Sigma BLA (□) and with added Ca (+)

The Sigma sample with 0.39 Ca^{2+} per protein molecule in 0.9 m*M* Na^{+} has $R = 1.17$. This value doesn't differ significantly from that of apo-BLA at this temperature. Increasing the Ca^{2+} concentration to 6.6 m*M* (1.2 Ca^{2+} per α-BLA) gives $R = 1.40$, indicating formation of a more stable conformer. In samples 3 and 4 the original Ca^{2+} content (1.8 m*M*) is maintained and the Na^{+} concentration is augmented. The increased R values for these samples prove that Na^{+} ions also have a stabilizing effect on BLA conformation.

4. pH-dependence

In Fig. 2 we present the results of the pH-dependence investigations of the tyrosine doublet intensity ratio. The α-lactalbumin was a Sigma sample containing 0.38 Ca^{2+} per protein molecule. Na^{+} ions were also present, which explains why the R-value is 1.4 at pH = 8, an indication of the folded structure of the molecule as explained in Sect. 2.

With decreasing pH, the intensity ratio decreases: at pH = 2 we found that the acid conformer has a low doublet intensity ratio which is comparable to that of the apo-conformer at pH = 8, namely 0.6. This experimental result is supported by recent ultraviolet Resonance Raman spectra of α-lactalbumin, obtained by Rava and Spiro (1985).

Comparing the intensities of the 830 cm^{-1} tyrosine peak at pH = 6.6 and pH = 2.1, they observed a slightly more intense peak at low pH, leading to a smaller intensity ratio. Within the framework of the Siamwiza theory this corresponds to a state where the phenolic hydroxyl group is acting as a proton

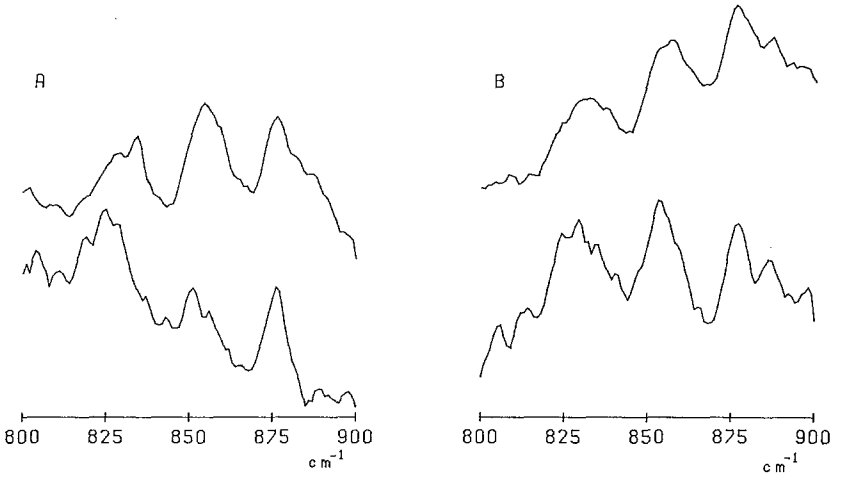

Fig. 3A and B. Spectra of BLA in the 800–900 cm^{-1} region. **A** Comparison of the spectra at pH = 8 *(top)* and pH = 2 *(bottom)* at room temperature. **B** Spectra at pH = 8 for BLA with Ca *(top)* and with EGTA *(bottom)*

donor and is forming moderate H-bonds in the protein interior. As proton acceptor candidates, carboxylate groups of the aspartic ($pK_A = 3.86$) and glutamic ($pK_A = 4.25$) acid residues have to be considered. In this way, the low intensity ratio for apo-troponin C is explained as consistent with the fact that one of the two tyrosines, Tyr 109, is located within a site rich in aspartic and glumatic acid residues, available for H-bonding (Carew et al. 1980). In our case however, at the lowest pH most of these groups are no longer negatively charged, so that H-bonding at this pH can only be understood if the protein undergoes a drastic conformational change whereby the $-COOH$ group is brought close to the tyrosine residue. The conformer at pH = 2 and the apo-conformer at pH = 8 possess an unfolded tertiary structure but an almost intact secondary structure (Hiraoka and Sugai 1985). Furthermore it has been suggested by Dolgikh et al. (1981) that the tertiary structure of the acid conformer slowly fluctuates. However, the fact that in a partially unfolded state Tyr groups are buried to a greater extent inside the protein remains surprising and contrasts with the tryptophan behaviour. From tryptophan fluorescence measurements (Hanssens et al. 1984; Permyakov et al. 1985) it is obvious that in the *U*-conformer, the tryptophan groups are more accessible to the solvent.

At high pH the ratio also decreases, to a value as low as 0.7 at pH = 11.6. This low value could be due to the alkaline denaturation of the protein. In their tyrosine titration experiments on BLA, Kuwajima et al. (1979) showed that the normalized absorption of the tyrosinate form at 298 mm increases from 0 to 1 in the pH range from 9 to 12. Analogous experiments by Permyakov et al. (1985) have confirmed these results and have demonstrated Ca^{2+} dependence of this transition curve for alkaline denaturation. The deprotonation of the tyrosyl side chains in this pH range is accompanied by a conformational change that favours a more expanded protein structure. In the Siamwiza theory, the ionization of the tyrosyl hydroxyl group is closely related to the reversal of the relative intensities of the Raman doublet so that low *R* values are obtained in the ionized state. In this ionized state we do not want to link the low *R* value with a low degree of exposure to the solvent, as done by Frushour and Koenig (1975) in their study of the alkaline denaturation of *β*-lactoglobulin. They argue that at high pH irreversible aggregation and polymerization occurred so that more tyrosines should be exposed to a hydrophobic environment, characteristic of the protein interior. We prefer to interpret this low value as corresponding to the ionization of the phenolic hydroxyl group in contact with the solvent. This interpretation is in agreement with the generally accepted denaturation mechanism whereby the protein is partially unfolded and contact between the side groups and the solvent is enhanced.

If this interpretation is correct, there should be a fundamental conformational difference, so fas as Tyr groups are concerned, between the acid and the alkaline denaturated protein. At the same time it would suggest that the straightforward interpretation, i.e. that low *R* values for proteins correspond to buried tyrosines and high values to exposed tyrosines, can be questioned. If the interpretation is limited to the charge state of the phenolic oxygen, the Siamwiza theory can be applied without modification to BLA.

5. *Chemical unfolding*

In the former experiments, we have interpreted a lower intensity ratio as an indication for the unfolding of the tertiary structure of the protein. The ratios obtained in experiments in the presence of chemical denaturants are interesting and decisive checks of these results. As a chemical denaturation agent, guanidine hydrochloride was used a pH = 8 and at room temperature. The *R* values of Table 2 indicate that BLA undergoes a gradual conformational change at increasing guanidine hydrochloride concentration. Here also the tyrosine residues seem to be less exposed to the solvent in the unfolded structure.

Conclusion

We have used the Raman doublet intensity of the tyrosine residues as an internal probe for the conformational state of BLA. The unfolding of the protein by chemical, thermal or alkaline denaturation can be monitored by comparing the Raman intensities of the 850 cm^{-1} and 830 cm^{-1} lines. The removal of Ca^{2+} ions as well as a low pH also leads to conformational changes that can be interpreted as unfolded structures. Addition of Ca^{2+} on the other hand stabilizes the protein.

If the interpretation of the Siamwiza theory that is established for the interpretation of this Raman

[Guanidine hydrochloride] (M)	R
0	1.30
1	1.27
2	1.08
3	1.04
4	0.88

Table 2. Effect of guanidine hydrochloride treatment on *R* at 23 °C and pH = 8

doublet in model compounds, can be used without modification for proteins, we came to the conclusion that the tyrosine residues move to the protein interior in the unfolded state. In contrast to the situation with tryptophan, the tyrosine groups in this case, will be less exposed to the solvent.

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