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PH-DEPENDENCE OF THE α-LACTALBUMIN STRUCTURE : A FLUORESCENCE STUDY

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SUMMARY : The fluorescence parameters of demetallized α-lactalbumin in the range from pH 8 to 2 show an extremum around pH 5-4 (a minimum in quantum yield and wavelength and a maximum in polarization). This extremum is not due to a competition between Ca^{2+} and protons but rather to a stabilization of the conformation of the protein near the isoelectric pH by the ionic interactions between local positive and negative charges on the protein. The calcium-free protein has similar fluorescence characteristics at pH 2 and 8 but the thermal transition curve is different. The influence of 0.1 M NaCl is also considered.

INTRODUCTION : It has been observed by Permyakov et al. (1) that in a mixture of bovine α-lactalbumin, Ca^{2+} and EGTA, the tryptophan fluorescence quantum yield decreases from pH 9 to 6. In the pH range from 6 to 4.5 the fluorescence reaches a minimum and increases again at lower pH values. To interprete these data, they assume that the Ca^{2+} binding properties of α-lactalbumin are independent of pH in the range from 9 to 6.5 while the Ca^{2+}-EGTA association constant diminishes from 10^{10} M^{-1} to 10^{4} M^{-1}. As a consequence, the decrease in quantum yield is caused by a redistribution of the Ca^{2+}-ions between EGTA and the protein. The conformational change at acidic pH is assumed to be caused by a competition of protons and Ca^{2+}-ions for the same site.

We obtained quantum yields of demetallized α-lactalbumin (apo-α-lactalbumin) as a function of pH and found the same minimum in the pH range from 4 to 5. Since there is no competition with Ca^{2+} in our experiments, another explanation for this behavior is suggested.

MATERIALS AND METHODS : Bovine α-lactalbumin is a Sigma product : 4 ml samples of 10 mg protein/ml are purified by gel chromatography at pH 5.3 on Sephadex G 100 (Ø = 2 cm, h = 50 cm). After dialysis against distilled water, the purified α-lactalbumin is demetallized by ion-exchange chromatography on Chelex 100. α-Lactalbumin solutions up to 0.8 mg/ml are obtained in that way. The protein concentrations are determined with a Beckman D-spectrophotometer at 280 nm using a value of $E_{1\%}$ = 20.1 (2). By atomic absorption the purified bovine α-lactalbumin preparations are found to be Ca^{2+}-free for at least 95 %. By the method of Murakami et al. (3) the protein is found to be metal-free for at least 85 %. Freshly purified protein solutions are used in all measurements. The solutions are diluted with HCl at

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pH 2 and 3 or appropriate buffers containing 0.005 M acetate (pH 4 and 5), 0.005 M MES (pH 6 and 7) or 0.005 M TRIS (pH 8). All buffer solutions are Chelex 100 treated. NaCl used in some experiments is a suprapure product from Merck.

Fluorescence measurements are performed with an Aminco SPF-500 spectro fluorimeter, connected with a Hewlett-Packard 7225 plotter and a Hewlett-Packard 9815 A disc-top computer. The latter calculates corrected spectra, the maximum wavelength and the area under the corrected emission spectrum. Excitation was made at 280 nm. Fluorescence quantum yields are evaluated by comparing the areas under the fluorescence spectra of protein preparations with those of an aqueous tryptophan solution (quantum yield 0.13 at 23°C) (4) with the same absorbance at the excitation wavelength.

Measurements of steady-state fluorescence polarization of tryptophan in α-lactalbumin are performed with the same instrument by using excitation and emission polarizers, according to the method of Azumi & McGlynn (5). The excitation wavelength was 280 nm and the emission wavelength for tryptophan was chosen at the maximum fluorescence intensity for each sample.

RESULTS AND DISCUSSION : Influence of Ca^{2+}-ions on the fluorescence parameters of α-lactalbumin

The effect of Ca^{2+} on the spectral characteristics of bovine α-lactalbumin at different pH values is shown in Table I. Within the range of pH 8 to 6, in presence of Ca^{2+}-ions the quantum yield of the protein is much smaller than in the metal-free medium and the emission band is shifted to shorter wavelengths. The polarization, on the contrary is about 10 % higher in the presence of Ca^{2+}. The pronounced decrease in fluorescence quantum yield and the spectral shift towards shorter wavelengths have been described by different authors (1,3,6). It has been described (6) that the fluorescence changes caused by the removal of Ca^{2+} at pH 7-8 closely

TABLE I

Comparison of the tryptophan fluorescence parameters for the α-lactalbumin (1.5×10^{-5}M) in the absence or presence of 3.8×10^{-5}M Ca^{2+} or 0.1 M NaCl at different pH-values, T = 23° C

	Quantum yield Q			Polarization values P			λ_{max} (nm)		
pH	apo-α-LA	2.5 Ca^{2+} / protein	0.1 M NaCl	apo-α-LA	2.5 Ca^{2+} / protein	0.1 M NaCl	apo-α-LA	2.5 Ca^{2+} / protein	0.1 M NaCl
8	0.051	0.032	0.036	0.092	0.102	0.102	337	325	325
7	0.047	0.032	0.034	0.092	0.105	0.104	334	325	325
6	0.043	0.030	0.033	0.095	0.105	0.105	331	325	325
5	0.033	0.028	0.030	0.104	0.104	0.104	327	325	325
4	0.032	0.027	0.036	0.102	0.102	0.098	329	325	331
3	0.042	0.040	0.042	0.091	0.091	0.093	337	334	337
2	0.046	0.046	0.045	0.094	0.095	0.092	339	338	341

resemble the changes that occur on bringing the Ca^{2+}-complexating protein (native protein) to pH 2. As seen from the table this is also confirmed by the polarization measurements. Therefore it was proposed (6) that these conversions cause a comparable structural change, and involve the loss of the most tightly bound calcium in the acidic medium. We provide evidence, however, in Figure 2, as will be seen further, that the fluorescence of the demetallized protein at pH 8 and pH 2 have not the same temperature dependence.

The main point in Table I and Figure 1 in comparison with previous work (1,3,6,7) is that the fluorescence parameters of the apo-α-lactalbumin itself are very pH-dependent. At pH 4 to 5 the calcium-free protein has a comparable emission spectrum, quantum yield and polarization value as the calcium protein at neutral pH. Murakami et al. (3) have outlined that fluorescence changes - caused by complexing α-lactalbumin with calcium - are not consistent with a direct contact quenching mechanism, but with a ligand - induced conformational change. According to these authors, the fluorescence characteristics indicate that tryptophan groups in calcium-free α-lactalbumin at pH 4 to 5 and in the native protein are in a comparable conformation. In that state tryptophans 28 and 109 are close to tryptophan 63, and part of their excitation energy is

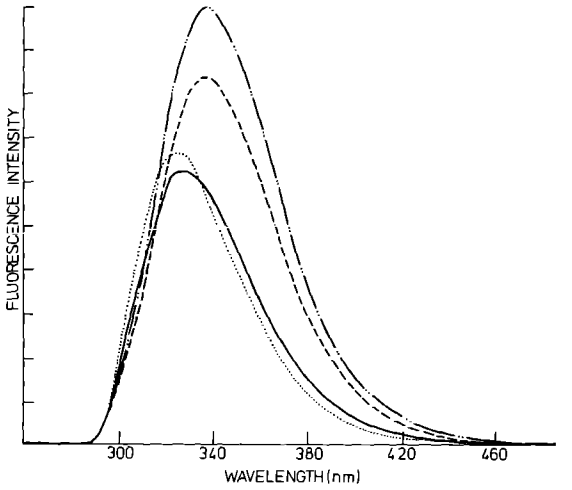

Figure 1. Comparison of corrected tryptophan emission spectra for demetallized bovine α-lactalbumin at pH 8 (—·—), pH 4 (——), pH 2 (----) and the same protein after addition of Ca^{2+}-ions at pH 8 (2.5 Ca^{2+}/protein)(....). Temperature 23° C. Protein concentration (1.5 10^{-5}M).

transferred to tryptophan 63, and further quenched as a result of the close proximity of disulfide bridges to that tryptophan group (7). Permyakov et al. (1) have observed that the fluorescence yield of a mixture of apo-protein, Ca^{2+} and EGTA decreases from pH 9 with a minimum in the pH region 4.5 to 6. Following their explanation the effect is due to a binding of Ca^{2+}-ions. From our measurements it is clear that it is mainly a property of the demetallized protein itself. Indeed, addition of Ca^{2+} to the apo-protein at pH 4 and 5 produces only a minor change in the fluorescence parameters as seen in Table I.

From sedimentation constants (8) and from electrophoretic mobilities (9) it was calculated that the apparent radius of Ca^{2+}-containing α-lactalbumin at pH2 is about 30 % larger than at pH8. In the compact conformation, the tryptophan quantum yield is smaller than in the expanded form (see table I). In the absence of complexating Ca^{2+}-ions, the fluorescence parameters of α-lactalbumin show an extremum around the iso-electric point, indicating that the changes in the configuration of the protein are determined by the electrostatic interactions. In that case, it is described by Tanford (10) that near the iso-electric point, the presence of an equal number of positive and negative charges will favor a compact configuration ; while, with an excess of positive or negative charges, the protein will possess a lower electrostatic free energy in the expanded configuration. Therefore the lower quantum yields of the apo-protein at pH4-5 (table I) are again related to a compact conformer and the higher quantum yields to an expanded form. As a consequence the increase in fluorescence seems primarily caused by an increase in the distance of one or more of the above mentioned tryptophan pairs that are directly or indirectly involved in the quenching process. Possible less favorable orientations between tryptophans may influence the result.

The lower polarization of the light emitted by the expanded protein is probably mainly due to a drastic increase of the mean fluorescence lifetime of the tryptophan residues corresponding to the increase of the

quantum yield. However a contribution of a possible higher mobility of some of the tryptophans cannot be excluded.

Temperature dependence of the fluorescence intensity of α-lactalbumin in the presence or absence of Ca^{2+}-ions.

Normally, with increasing temperature the fluorescence intensity decreases due to thermal quenching. However, Sommers and Kronman (7) have shown that thermally induced transitions in bovine α-lactalbumin can be observed from plots of the fluorescence intensity at 350 nm versus temperature. Fig. 2A shows these plots of the demetallized α-lactalbumin at pH 8, 4 and 2. At pH 8 the protein undergoes a conformational transition in the room temperature region. At pH 4 the fluorescence intensity increases up to 40°C while at pH 2 the high fluorescence intensity decreases in the whole temperature range but no conformational transition is observed. These results suggest that in spite of the fact that the spectral characteristics of Ca^{2+}-free α-lactalbumin at pH 8 and 2 are very similar, the stability of their conformation is different.

In figure 2B it is shown that at pH 8, addition of a small excess of Ca^{2+}-ions shifts the transition from room temperature to the 50-60°C region.

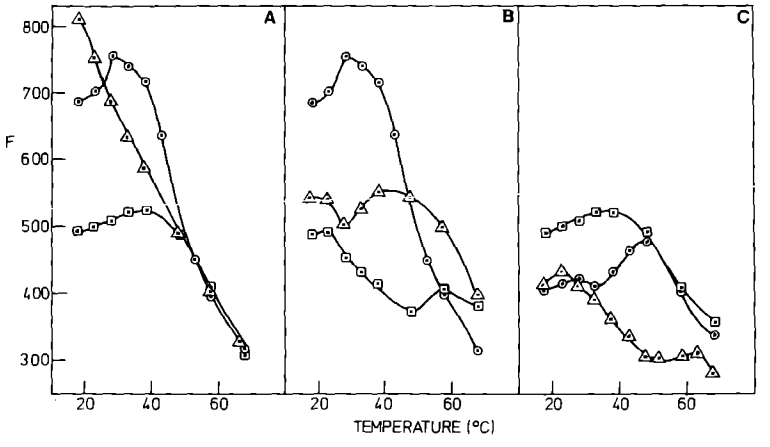

Figure 2. Temperature dependence of the fluorescence intensity F at 350 nm for apo α-lactalbumin in different conditions of pH and ion concentrations.
A. pH 8 ⊙ 4 ⊡ 2 △ (no Ca^{2+} or NaCl)
B. at pH 8 ⊙ no Ca^{2+} or NaCl △ 0.1 M NaCl ⊡ 2.5 Ca^{2+}
C. at pH 4 ⊡ no Ca^{2+} ⊙ 4.10^{-5}M Ca^{2+} △ 10^{-1}M Ca^{2+}

Therefore, in agreement with an earlier observation (11), Ca^{2+}-ions stabilize the α-lactalbumin structure.

Although one can conclude from Table I that the tryptophan groups in calcium-free α-lactalbumin at pH 4 are in a comparable conformation as in the native protein at pH 8, the data of Fig. 2C show that even at pH 4, this conformation can be further stabilized by the addition of an excess Ca^{2+}. Indeed, the transition in the 20°-40° C region for the calcium-free protein shifts to 35°-50° C for 2.5 Ca^{2+} protein, and to 50°-60° C at 0.1 M Ca^{2+}, which is the same transition region as the native protein at pH 8 (7, 11, Fig. 2B). The result can be explained by accepting a competitive replacement of protons by Ca^{2+}-ions in the original calcium binding site.

pH-dependence in the presence of NaCl

At 23° C and different pH values, the presence of 0.1 M NaCl influences the tryptophan fluorescence of α-lactalbumin in a manner which is very similar to that of Ca^{2+}-ions. A similar effect on the demetallized protein was observed by Hiraoka et al. (11) with 0.2 M KCl by circular dichroism. From the NaCl-product label it is clear that this effect cannot be caused by the presence of divalent ion impurities. Therefore the effect of the ionic strength must be considered.

A high ionic strength decreases the interaction between the positive and the negative charges at different places on the protein. Such decrease of the ionic interaction at pH 8 means a decrease of repulsion between negative charges. Around the isoelectric pH, the attractive forces stabilizing the conformation are suppressed by the Na^+ and Cl^- ions, resulting in a small increase in distance between the tryptophan residues. The fact that 0.1 M NaCl shows no influence on the quantum yield of apo-α-lactalbumin at pH 2 is less clear, since one expects the same effect as at pH 8. One reason that this change does not occur may arise from the fact that the conformation of the protein at pH 8 shows a thermal transition at room temperature (Fig. 2A) while the pH 2 conformation does not.

Also Kuwajima et al. (12) have outlined that a total unfolding of α-lactalbumin needs a higher concentration of guanidine hydrochloride in an acidic medium than at neutral pH.

CONCLUSION : The fluorescence parameters of the Ca^{2+}-free α-lactalbumin around the iso-electric point (pH 4-5) are comparable to that of the Ca^{2+}-bound protein at neutral pH. The parameters of the apo-protein are influenced in a same way by an excess of positive charges (at pH 2) as by an excess of negative charges (at pH 8).

The relative low fluorescence quantum yields and high polarization values are related to compact conformations of the protein.

The temperature dependence of the fluorescence spectra shows that comparable conformations have different thermal stability.

The ionic strength of the solution has a more important effect on the conformation of the protein at neutral pH than at acidic pH.

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