Biophysical Chemistry 15 (1982) 19–26
Elsevier Biomedical Press

INTRINSIC FLUORESCENCE SPECTRA OF A TRYPTOPHAN-CONTAINING PARVALBUMIN AS A FUNCTION OF THERMAL, pH AND UREA DENATURATION

Eugene A. PERMYAKOV [a], Vladimir V. YARMOLENKO [a], Edward A. BURSTEIN [a] and Charles GERDAY [b]

[a] Institute of Biological Physics, U.S.S.R. Academy of Sciences, 142292 Pushchino, Moscow Region, U.S.S.R. and [b] Laboratoire de Biochimie Musculaire, Institut de Chimie Organique et de Biochimie, Université de Liège au Sart Tilman, B-4000 Liege, Belgium

Received 27th May 1981
Revised manuscript received 17th September 1981

Key words: Tryptophan fluorescence; Denaturation; Parvalbumin; (Whiting)

The thermal, pH and urea denaturation of the calcium-loaded protein from whiting has been studied by means of the intrinsic fluorescence of the single tryptophan residue. pH denaturation of the protein takes place at a pH greater than 11.5 and lower than 5.5. Thermal denaturation of the protein occurs at temperatures above 55°C. Urea initiates the denaturation of the calcium-loaded protein at rather low concentrations (1.0 M). In all cases, whether pH, thermal or urea denaturation, intermediate states of the protein were recorded. The fluorescence spectra of these intermediates are similar to that of the protein with one equivalent of calcium bound. Whiting parvalbumin binds calcium in the presence of 7.5 M urea but under these conditions, calcium-binding constants of the protein have been shown to be $10^2-10^3\ M^{-1}$ (in comparison with 5×10^8 and $6\times10^6\ M^{-1}$ in the absence of any denaturing agents).

1. Introduction

Parvalbumins are a well known family of calcium-binding proteins (M_r 10000–13000) found in various concentrations in the skeletal muscles of vertebrates [1]. The crystal structure of carp parvalbumin at p*I* 4.25 has been determined and refined to 1.9 Å resolution [2]. The molecule contains six helical regions, designated A–F, and binds strongly two Ca^{2+}: one in the loop between helices C and D (CS-site) and the other in the loop between helices E and F (EF-site). The structure of calcium-loaded parvalbumin is rather stable. According to the fluorescence and circular dichroism data, calcium-loaded parvalbumins retain their native structure within a wide pH range from approx. 5 to 10–11 [3,4]. Only weak changes in the CD spectra in the far-ultraviolet region occur following acidification [4]. This result is in agreement with calorimetric [5] and fluorescence [6] data and suggests that the protein is still largely ordered at acidic pH. The near-ultraviolet CD spectra of parvalbumins are considerably modified at pH values below 4 [4]. The abrupt changes occurring around pH 3.7 seem to be due to the titration of carboxyl groups. Alkaline denaturation (pH > 11) causes an increase in the accessibility of phenylalanine and tyrosine residues to water [3] and a decrease in the protein affinity for calcium [7]. ^{43}Ca-NMR studies, however, indicate that parvalbumin is able to interact with calcium even at very high pH values [7].

According to NMR [8], calorimetry [5] and fluorescence [3,6,9] data, the thermal denaturation of calcium-loaded parvalbumins occurs at temperatures above 50–60°C. The removal of tightly bound calcium results in a drastic decrease in protein stability and the enthalpy of denaturation [5]. According to optical rotatory dispersion data [10], the helical content of the calcium-loaded parvalbumin from carp at p*I* 3.95 is practically

Abbreviations: SDS, sodium dodecyl sulphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid.

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invariant at 25, 50 and 83°C. This indicates that the tertiary structure of parvalbumin can be significantly disordered, as shown by NMR [7,8] and intrinsic fluorescence [3], without apparent significant changes in its secondary structure.

Urea, guanidine hydrochloride and SDS denature calcium-loaded parvalbumins [4]. Effects on the dichroic signals of the calcium-loaded parvalbumins from pike an whiting are not detected before the urea concentration reaches 4–5 M, whereas full denaturation occurs in 8 M urea. Incubation of pike parvalbumin in 0.1 M SDS results in a conformational alteration leading to exposure of the aromatic side chains to the solvent but with an almost unchanged helical content [4]. The transition midpoints for the denaturation of carp parvalbumins by guanidine hydrochloride are located at 1.8–2.2 M as shown by CD and fluorescence spectroscopy [6].

In this paper, we present the results of a more detailed study of pH, thermal and urea denaturation-processes of parvalbumin. The aim of the study was to detect any possible correlations between denaturation and calcium binding by comparing states of the protein induced by denaturation processes with those already published [9] corresponding to the protein with different calcium contents. The study was carried out by means of intrinsic protein fluorescence techniques. Thus far, most fluorescence studies on parvalbumins have been carried out on proteins devoid of tryptophan [3,6]; it is well known, however, that the fluorescence properties of tryptophan residues are much more sensitive to the chromophore environment than those of phenylalanine and tyrosine residues [11]. For this reason, the existence of a single tryptophan residue (Trp-102) in whiting parvalbumin [12] gave us the chance to obtain by means of its fluorescence more detailed information about the physico-chemical peculiarities of this parvalbumin.

2. Materials and methods

The major component (p*I* 4.44) of whiting (*Gadus merlangus*) parvalbumin was prepared as described earlier [12]. The purity of the protein preparation was checked by polyacrylamide gel electrophoresis and ultraviolet absorption spectra carried out with 0.01–0.1 mM parvalbumin solutions in 0.05 M Tris-Hepes buffer, pH 7.5. Protein concentrations were evaluated spectrophotometrically (Specord UV-VIS, Karl Zeiss, Jena, G.D.R.) using the molar absorption coefficient $\epsilon_{280\ nm} = 7400\ M^{-1}\ cm^{-1}$ [12]. All chemicals used were of analytical grade.

Fluorescence spectra were recorded from the front cell surface using a laboratory-made spectrofluorimeter described earlier [13], equipped with spectral slit widths of about 1 nm. Protein fluorescence was excited at 280.4 nm. All fluorescence spectra were corrected for the instrumental spectral sensitivity. Intensities in corrected spectra are proportional to the number of photons emitted in the unit time interval per unit wavelength interval. The reproducibility of intensity measurements was better than 3%. Fluorescence quantum yield was evaluated by comparing the area under the fluorescence spectrum of a protein sample with that of an aqueous tryptophan solution (quantum yield 0.23 at 20°C) [14] of the same absorbance at the excitation wavelength. Since the fluorescence spectrum of the calcium-loaded parvalbumin from whiting possesses elements of vibrational structure [9,14], the position of the middle of a chord, drawn at the 80% level of the maximal intensity ($\bar{\lambda}$), was taken as a measure of the spectrum position. The intensity of the mercury line emission at 313.2 nm, scattered by the protein solution at about 150° from the direction of the excitation beam, was used to monitor eventual aggregation processes in protein solutions.

The temperature dependence of fluorescence spectrum characteristics was investigated using thermostatically controlled water circulating in the hollow brass cell-holder. The temperature in the sample cell was monitored by means of an MT-54 microthermoresistor.

pH values of solutions were measured to an accuracy of ±0.05 pH units. In titration experiments, pH values were adjusted by means of a polyethylene rod slightly moistened with either 0.1 N HCl or 0.1 N KOH.

3. Results

3.1. Acidic and alkaline denaturation

Fig. 1 depicts the pH dependence at 20°C of the fluorescence spectrum characteristics of whiting parvalbumin (the solution contained approx. 2.8 mol calcium/mol protein). In the pH range 5.5–9.5 all the parameters remain essentially constant. The 30% drop in the quantum yield value between pH 9.5 and 11.5 could be due to titration of the phenolic -OH group of Tyr-26 [4] and thus eventually to a long-distance tryptophan-tyrosine energy transfer. A spectral shift towards longer wavelengths along with further reduction of the quantum yield are observed at pH 11.5 [16].

In the acidic part of the plots, two pronounced changes in fluorescence parameters take place around pH 5.1 and 3.5. The first probably reflects some loosening of the protein structure induced by protonation of carboxyl groups lowering their affinity for calcium. This is confirmed by the position (326.5 nm) of the fluorescence spectrum at pH 4.3 (inset of fig. 1), which indicates a buried location of the tryptophan residue but in a less rigid environment than at neutral pH values [11]. The spectrum coincides practically with that at neutral pH; i.e., protein containing only one bound Ca^{2+}, presumably at the CD-site [9]. This may mean that the acidic denaturation of whiting parvalbumin is a complex, successive process beginning with neutralization of the carboxyl groups in one of the calcium-binding domains, most likely the EF-loop.

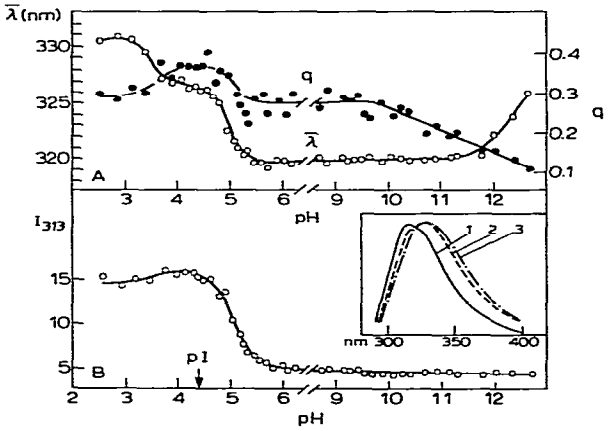

Fig. 1. (A) pH dependence of the effective spectrum position, $\bar{\lambda}$, and fluorescence quantum yield, q, and (B) the intensity of the scattered light of the mercury line at 313 nm for calcium-loaded whiting parvalbumin. Inset: fluorescence spectra of whiting parvalbumin at different pH values: (1) pH 7.0, (2) pH 4.3 (intermediate state), (3) pH 2.6. Fluorescence excited at 280.4 nm. Protein concentration 61 μM; 2.8 mol calcium/mol protein; 20°C.

The first spectral change is accompanied by an isoelectric aggregation of the protein (pI 4.44) reflected in the increase in light scattering (fig. 1B). The possibility cannot excluded that the quantum yield enhancement in this pH range is an artifact produced by light scattering so that any interpretation from this parameter could be misleading.

The second spectral change (pH 3.75–3.25) seems to be due to a further denaturation stage induced by protonation of another class of carboxyl group, presumably coordinating calcium at the CD-site. The position of the protein fluorescence spectrum at approx. 331 nm and its half-width of approx. 55 nm at pH 2.6 (inset in fig. 1, curve 3) still suggest an internal location of the tryptophan residue in the protein molecule [11]. Aggregation of the protein in this pH region (fig. 1B), though less intense than that at pH 4, could prevent transfer of Trp-102 to an aqueous environment.

3.2. Thermal denaturation

Heating of the calcium-loaded parvalbumin solution (2.2 mol calcium/mol protein, pH 6.7) from 7.5 to about 55°C does not significantly change the fluorescence spectrum position (fig. 2A). From 55 to 92°C, a shift of the spectrum by about 20 nm towards longer wavelengths occurs; this is accompanied by a slight deflection in the quantum yield versus temperature curve. Fig. 2B shows a phase plot of fluorescence intensity at 360 nm versus that at 320 nm [9,11]. For a simple transition between two states A and B, the phase plot $I(\lambda_1)$ versus $I(\lambda_2)$ should appear as a segment of the straight line joining the points corresponding to the spectra of the extreme states A

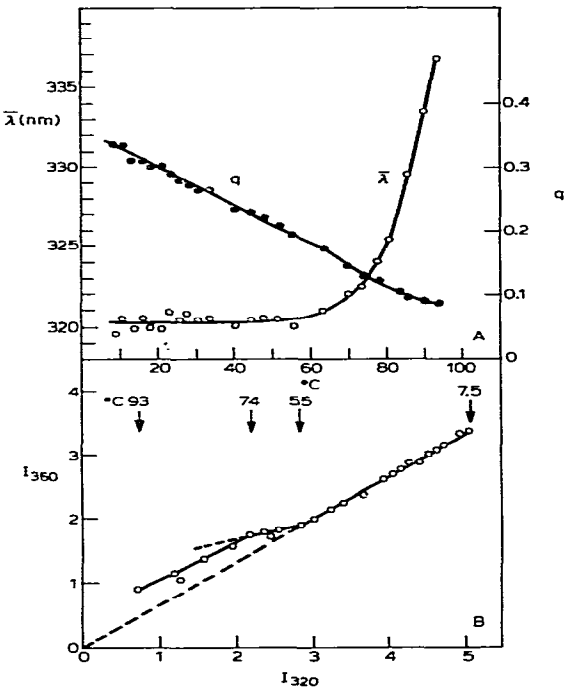

Fig. 2. (A) Temperature dependence of the spectrum position, $\bar{\lambda}$, and the fluorescence quantum yield, q, of whiting parvalbumin. (B) Fluorescence phase plot corresponding to the temperature dependence of the protein fluorescence (values of I are expressed in relative units). Protein concentration 50 μM; 2.2 mol calcium/mol protein; 0.05 M Tris-Hepes, pH 6.8. Fluorescence excited at 280.4 nm.

and B. In a more complex case, where the transition passes through an intermediate D (A $\rightleftharpoons$ D $\rightleftharpoons$ B) differing in its fluorescence spectrum from A and B, the plot of $I(\lambda_1)$ versus $I(\lambda_2)$ should show a more or less pronounced bend depending on the contribution of the intermediate to the total emission and on the extent of the difference in its fluorescence properties from those of A and B. On the phase plot (fig. 2B), three distinct parts are visible. The first portion (7.5–55°C), which can be extrapolated to the origin, corresponds to ordinary thermal quenching, without any changes in the shape or position of the spectrum, due to the thermal activation of intramolecular collisions between excited indole groups and neighboring quenching groups [11,18]. The second (55–74°C) and third (>74°C) linear parts seem to correspond to at least two successive conformational changes affecting the structure around the tryptophan residue. The existence of the two segments in the phase plot indicates that the denaturation process passes through an intermediate the maximal population of which is observed at about 74°C under our conditions. The fluorescence spectrum of the protein at 74°C is presented in fig. 3 (curve 2). The parameters of this spectrum correspond to interior localization of Trp-102. The spectrum is similar to that of the mono-calcium form of whiting parvalbumin [9]. Since the system is heated by circulating water, it is not possible to achieve complete denaturation of the protein which is expected to occur at temperatures above 100°C. The fluorescence spectrum of whiting parvalbumin at 93°C is also shown in fig. 3 (curve 3). Its position ($\approx$ 337 nm) and large width show that it contains components corresponding to the emission of a completely exposed tryptophan residue [11].

3.3. Urea denaturation

An increase in urea concentration results in very pronounced spectral changes (fig. 4). In 7.5 M urea, the fluorescence spectrum is shifted towards longer wavelengths by 33–34 nm in comparison with that of the native protein, and its position at 353 nm coincides with that of the spectrum of free

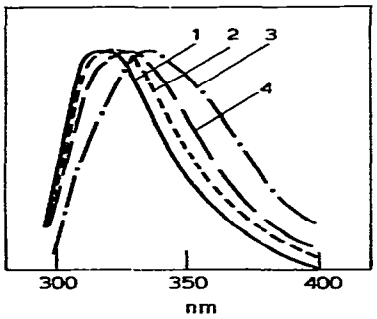

Fig. 3. Fluorescence spectra of whiting parvalbumin at different temperatures: (1) 55°C, (2) 74°C (intermediate state), (3) 93°C. Conditions as in fig. 2. (4) Fluorescence spectrum of whiting parvalbumin containing one bound Ca^{2+}.

aqueous tryptophan [11]. It is worth noting that calcium-free parvalbumin obtained in the presence of a great excess of EGTA has a fluorescence spectrum position at 346 nm [9]. This means that the structure of aqueous calcium-free parvalbumin is somewhat more compact than that obtained in the presence of 7.5 M urea.

The main part of the spectral shift is observed within the urea concentration range from about 4 to about 6 M. However, the pronounced quantum yield change, accompanied by a moderate spectral shift and widening, occurs at lower urea concentrations. It has been shown that effects on the CD signals of whiting parvalbumin are not detectable before the urea concentration reaches 4–5 M, whereas full denaturation takes place in 8 M urea [4]. The two-step quantum yield decrease (fig. 4B) indicates the existence of an intermediate state of the protein. The fluorescence phase plot supports the idea of an intermediate at a urea concentration of about 3.2 M (fig. 5A). The fluorescence spectrum of the protein in 3.2 M urea is shown in fig. 5B (curve 2). It is quite similar to that of the mono-calcium form of parvalbumin in aqueous solution (curve 4) as well as to those of the intermediates observed in acidic and thermal denaturation processes.

In the urea concentration range 6–7.5 M there is another change in the tryptophan environment which results in a smooth bending of the phase curve (fig. 5A) and in an increase in the fluorescence quantum yield (fig. 4B). The nature of this change is not sufficiently clear at present. This could reflect additional binding of urea to the denatured protein.

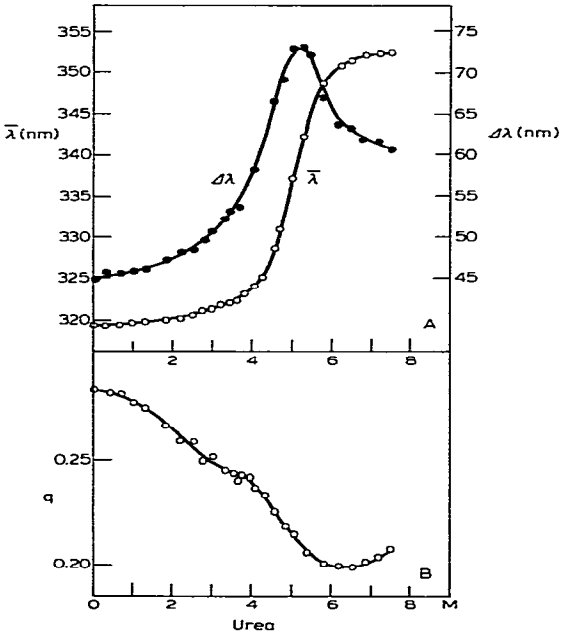

Fig. 4. Dependence of the fluorescence parameters of whiting parvalbumin on urea concentration: (A) spectrum position, $\bar{\lambda}$, and width, $\Delta\lambda$; (B) fluorescence quantum yield. Fluorescence excited at 280.4 nm. Protein concentration 48.8 μM; 2.2 mol calcium/mol protein; 20°C; 0.05 M Tris-Hepes, pH 7.5.

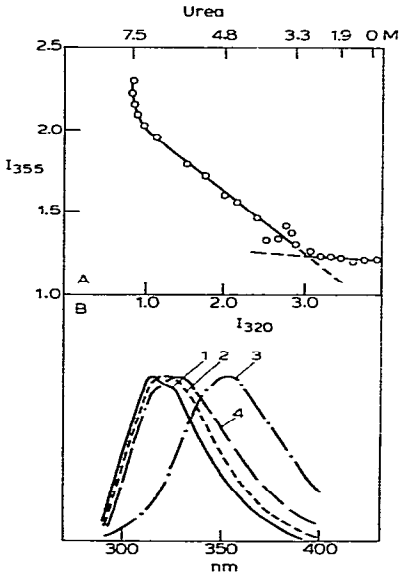

Fig. 5. (A) Fluorescence phase plot corresponding to the dependence of parvalbumin fluorescence on urea concentration (fig. 4). Values of I are expressed in relative units. (B) Fluorescence spectra of whiting parvalbumin at different urea concentrations: (1) 0 M, (2) 3.2 M (intermediate state), (3) 7.5 M. Conditions as in fig. 4. (4) Fluorescence spectrum of whiting parvalbumin containing one bound Ca^{2+}.

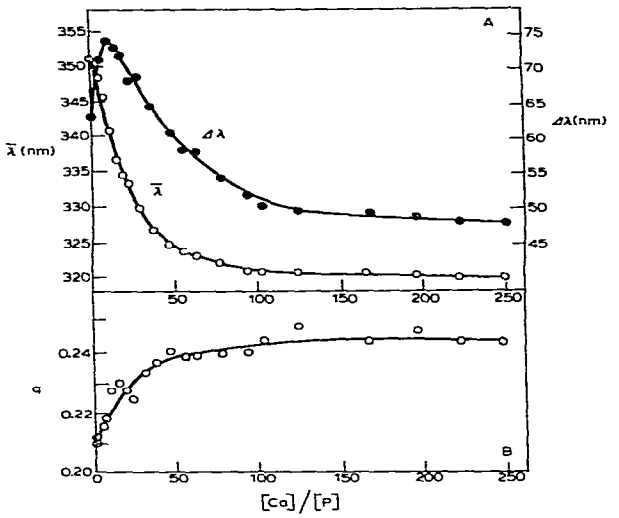

Fig. 6. Titration of whiting parvalbumin with calcium in the presence of 7.5 M urea. (A) spectrum position, $\bar{\lambda}$, and width, $\Delta\lambda$; (B) fluorescence quantum yield, q. Fluorescence excited at 280.4 nm. Protein concentration ([P]) = 48 μM.

3.4. Calcium binding in 7.5 M urea

The results of the calcium titration of whiting parvalbumin in the presence of 7.5 M urea are presented in fig. 6. The parameters $\bar{\lambda}$, $\Delta\lambda$ and q are plotted versus the ratio of the total calcium concentration ([Ca]) to protein concentration ([P]). The gradual increase in the calcium concentration produces changes in the fluorescence parameters

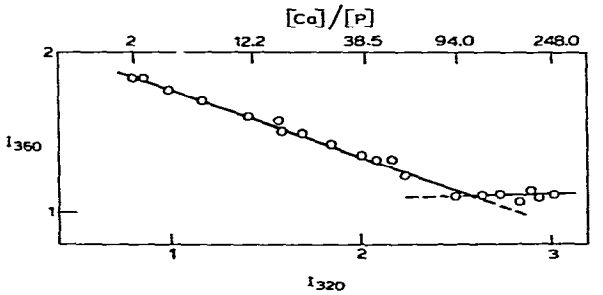

Fig. 7. Fluorescence phase plot corresponding to calcium titration of the protein in the presence of 7.5 M urea (fig. 6). Values of I are expressed in relative units.

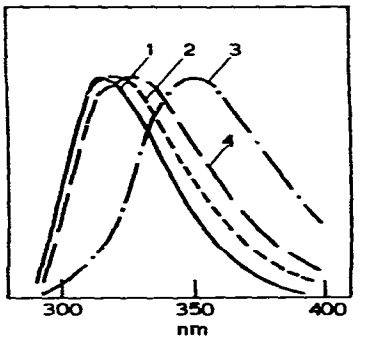

Fig. 8. Fluorescence spectra of whiting parvalbumin at different calcium concentrations in the presence of 7.5 M urea: (1) [Ca]/[P] = 248, (2) [Ca]/[P] = 94 (intermediate state), (3) [Ca]/[P] = 2. Conditions as in fig. 6. (4) Fluorescence spectrum of the protein containing one bound Ca^{2+} in the absence of any denaturing agents.

which are opposite to those induced by an increase in urea concentration. However, we could hardly approach the characteristics of the spectrum inherent to the two-calcium state of parvalbumin in the absence of urea even at very high calcium concentration (≈ 12 mM). The plots reach a plateau at [Ca]/[P] ≈ 100. This indicates weak calcium binding constants under such conditions. Corresponding curves for native aqueous whiting parvalbumin approach a plateau at [Ca]/[P] ≃ 2 [9]. Fig. 7 presents the fluorescence phase plot corresponding to the calcium titration of parvalbumin in 7.5 M urea. The phase plot demonstrates the existence of at least one intermediate state of the protein during this titration. Its fluorescence spectrum is shown in fig. 8 (curve 2). It is also rather similar to that of the mono-calcium form of the protein (curve 4).

4. Discussion

As shown earlier [9], the tryptophan fluorescence spectrum of the calcium-loaded parvalbumin from whiting at neutral pH values and at room temperature is positioned at very short wavelengths with a maximum at 315.5 nm (fig. 5, curve 1). Such a spectral feature favours the idea that in the calcium-loaded molecule, Trp-102 is

buried in a weakly polar and rigid (the dipole relaxation time is much longer than the fluorescence lifetime) environment [11]. Only one protein demonstrates a shorter wavelength position of the tryptophan fluorescence spectrum, namely, azurin [13], which at ordinary temperatures, also has a well resolved fine structure with a main maximum at 306.5 nm. In this protein, tryptophan is located in an unusual environment characterized by the absence of hydrogen bonding or other polar interactions. However, such interactions (exiplex formation) may exist in whiting parvalbumin. In the present work, it has been shown that the acidic, thermal and urea denaturation of whiting parvalbumin passes through intermediate states having very similar fluorescence spectra which, moreover, practically coincide with those of the mono-calcium form of the protein observed at neutral pH under non-denaturing conditions. Such intermediates were not observed in the case of alkaline denaturation of the proteins which seems to be induced by the deprotonation at high pH values of the key guanidinium group of the single Arg-75 residue which with Glu-81 forms an internal salt bridge essential for maintaining the native structural integrity of the protein [15,16]. Following calcium titration, the protein in 7.5 M urea passes through a state having a fluorescence spectrum also reminiscent of that of the mono-calcium parvalbumin (fig. 8). One can assume that under these conditions and at a high calcium concentration, two Ca^{2+} can also be bound to the parvalbumin molecule as in the absence of urea, the difference being that the affinities of the two sites for calcium are much lower and of the order of 10^3 and $10^2\ M^{-1}$, respectively.

Such a similarity between the characteristics of the native mono-calcium form and of the intermediates observed under denaturing conditions indicates that the first stage of the denaturation processes does not grossly distort the protein structure but, rather, only diminishes the calcium-binding affinity of the presumably weaker EF-site. As a result, at relatively low calcium concentrations, the protein loses one of the bound Ca^{2+} and undergoes a structural transition (loosening of the structure), apparently similar under different conditions (temperature, urea or acid). This view is supported by the fact that even in 7.5 M urea a great excess of calcium is able to reverse the urea denaturation effect.

However, it is difficult using fluorescence data to reveal the exact nature of the parvalbumin intermediate states only on the basis of the intrinsic fluorescence of Trp-102 which is only sensitive to the organization of its immediate environment [11]. In the case of carp parvalbumin at p*I* 4.25, the data obtained from scanning calorimetry [5] have illustrated that at $pH < 6$, the thermal denaturation is rather complex, showing in particular superimposed peaks of the heat absorption. This means that despite similar environments of Trp-102, the structure of the possible intermediates of whiting parvalbumin under various conditions could be different.

It is interesting to compare the results of our study of the urea denaturation of parvalbumin with those of troponin-C [19]. The addition of 6 M urea in the presence of EGTA causes an unfolding of calcium-sensitive α-helical regions of skeletal muscle troponin-C illustrated by changes in the far-ultraviolet CD spectra. Further titration of the solution with calcium produces changes in the ellipticity and tyrosine fluorescence which increase linearly up to about 1 mol calcium added/mol (instead of 2 mol/mol in the absence of urea). It was suggested that in the presence of urea, the binding of calcium to one, presumably high-affinity site, produces essentially all the CD and fluorescence changes that are observed under native conditions by the binding of two Ca^{2+}/molecule of troponin-C. The domains affected by urea appear to be other than those affected by Ca^{2+} and represent α-helical segments stable in the native state in the absence of calcium. The calcium-binding constants of troponin-C in the presence of urea were not evaluated, but from the figures presented it is obvious that they are much higher than those of whiting parvalbumin in the presence of urea.

Data from scanning microcalorimetry [20] also suggest increased thermal stability of the troponin-C structure in comparison with that of whiting parvalbumin. In a medium containing excess calcium, the protein structure can be represented by two practically independent cooperative blocks;

one containing Ca^{2+}-specific binding sites melts at about 90°C (like parvalbumin) and the other containing Ca^{2+}- or Mg^{2+}-binding sites remains unmelted up to 110°C.

Thus, in spite of the structural homology of troponin-C and parvalbumin, some physicochemical properties, namely the stability of their structures towards urea and thermal denaturation, are different.

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