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SOME ASPECTS OF STUDIES OF THERMAL TRANSITIONS IN PROTEINS BY MEANS OF THEIR INTRINSIC FLUORESCENCE

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The changes in intrinsic fluorescence parameters induced by thermal transitions in proteins are developed on the background of the common thermal fluorescence quenching due to an activation of collisions between the excited chromophores and neighbouring quenching groups. Two methods of separation of the thermal quenching and conformational change contributions to the temperature dependence of the fluorescence parameters are presented. One is based on the use of the linearity of the plots of the reciprocal fluorescence quantum yield, $1/q$, vs. the T/η ratio (T, temperature; η, solvent viscosity) for native proteins containing a single fluorescing chromophore (T.L. Bushueva, E.P. Busel and E.A. Burstein, Biochim. Biophys. Acta 534 (1978) 141). The other method is based on a consideration of the phase plots for the tryptophan fluorescence of proteins (fluorescence intensity at a fixed wavelength vs. intensity at any other fixed wavelength). The methods have been used for a study of the thermal transitions in Mg^{2+}-loaded whiting parvalbumin (tryptophan fluorescence), Mg^{2+}-loaded pike parvalbumins pI 4.2 (tyrosine fluorescence) and pI 5.0 (phenylalanine fluorescence), and Ca^{2+}-loaded bovine α-lactalbumin (tryptophan fluorescence). The thermal denaturation curves for the parvalbumins show two-stepped character. The main change of the protein conformation occurs at the higher temperature step. Comparison of the fluorescence data with the microcalorimetry results shows that the maxima of the asymmetric heat sorption peaks for pike parvalbumins correlate with the mid-points of the higher temperature steps of the fluorimetric curves.

1. Introduction

Studies of thermal transitions in proteins provide valuable information about their structural parameters. Besides direct calorimetric measurements, many other physico-chemical methods are used for such studies. One of the most frequently used procedures is the intrinsic fluorescence method. The temperature-induced denaturation changes in a protein molecule usually result in increased exposure of the protein chromophores (tryptophan, tyrosine and phenylalanine residues) to water. This causes changes in their fluorescence. The most distinct changes occur in the case of tryptophan fluorescence. As a rule, the thermal denaturation of a protein results both in a change of its tryptophan fluorescence quantum yield and in a shift of its tryptophan fluorescence spectrum towards longer wavelengths. In the cases of tyrosine and phenylalanine fluorescence, protein denaturation results only in a change of the fluorescence quantum yield [1].

A quantitative consideration of the thermal denaturation process requires the use of fluorescence parameters which are a linear measure of the extent of denaturation conversion. Such parameters may be, for instance, the fluorescence quantum yield or the fluorescence intensity at a fixed wavelength [1]. However, the changes in these parameters, induced by thermal denaturation, are developed on the background of a common decrease in the fluorescence quantum yield due to an

activation of collisions of the protein chromophores with neighbouring quenching groups (the thermal quenching process) [1–3]. This complicates quantitative analysis of the experimental fluorescence curves and forces us to look for a technique of separation of the thermal quenching and conformational change contributions to the temperature dependence of the fluorescence parameters.

Here we present two such methods, one of which is of use for tryptophan, tyrosine and phenylalanine fluorescence of proteins whereas the other is suitable for tryptophan fluorescence only. The first method is based on the use of the linearity of the plots of reciprocal fluorescence quantum yield, $1/q$, against T/η (T, temperature; η, solvent viscosity), revealed by Bushueva et al. [2,3] for native proteins containing a single emitting chromophore. The second method is based on consideration of the phase plots for the tryptophan fluorescence of proteins (fluorescence intensity at a fixed wavelength vs. intensity at any other fixed wavelength). These methods seem to be convenient not only for intrinsic protein fluorescence but also in the cases of measurements of the emission of tightly bound fluorescent probes, labels and cofactors.

2. Materials and methods

Fluorescence measurements were performed with a laboratory-built spectrofluorimeter described earlier [4]. The fluorescence was collected from the front surface of the cell. All fluorescence spectra were corrected for the instrumental spectral sensitivity. Intensities of the corrected spectra are proportional to the number of photons emitted per unit wavelength interval. Protein fluorescence quantum yield was evaluated by comparing the areas under the fluorescence spectra of a protein sample with that of aqueous tryptophan (quantum yield 0.23 at 20°C [5]), tyrosine (quantum yield 0.20 at 20°C [5]) or phenylalanine (quantum yield 0.038 at 20°C [5]) solutions with the same absorbance at the excitation wavelength. The excitation wavelengths are indicated in the figure legends.

The temperature in the thermostatted cell of the instrument was measured by means of a copper-constantan thermocouple with an accuracy of approx. 1 K. The heating rate was approx. 1 K min^{-1}.

Calorimetric measurements were performed in a DASM-1M adiabatic scanning microcalorimeter with 1 ml cell volume and a heating rate of 1 K min^{-1}. The transition enthalpy was determined from the heat sorption peak above the lines extrapolating heat capacities of native and denatured states to the melting temperature [6].

Fitting of the experimental data with theoretical curves was carried out with an M-4030 computer using a non-linear regression scheme (Marquardt's algorithm) [7].

The study was carried out with bovine α-lactalbumin and three different fish parvalbumins. Pure preparations of the proteins were isolated and generously supplied to us by three researchers: whiting parvalbumin pI 4.44 by Dr. C. Gerday from Laboratoire de Biochimie Musculaire, Institut de Chimie Organique et de Biochimie, Université de Liège, Liège, Belgium; pike parvalbumins pI 4.2 and 5.0 by Dr. V.N. Medvedkin from the Institute of Protein Research of the U.S.S.R. Academy of Sciences, Pushchino, Moscow Region, U.S.S.R.; and bovine α-lactalbumin by Dr. V.V. Yarmolenko from the Lithuanian Branch of the All-Union Research Institute of the Butter and Cheese Industry, Kaunas, U.S.S.R. The purity of the protein preparations was checked electrophoretically, spectrophotometrically and spectrofluorometrically. Protein concentrations were evaluated spectrophotometrically using molar absorption coefficients of $\epsilon_{280} = 7400$ M^{-1} cm^{-1} for whiting parvalbumin [8], $\epsilon_{257} = 2699$ M^{-1} cm^{-1} for pike parvalbumin pI 4.2 [9]. $\epsilon_{259} =$ 1810 m^{-1} cm^{-1} for pike parvalbumin pI 5.0 [10] and $\epsilon_{280} =$ 28542 M^{-1} cm^{-1} for bovine α-lactalbumin [11].

Ultraviolet absorption spectra were registered with a Specord UV-VIS spectrophotometer (Carl Zeiss, Jena).

All solutions were made using deionised water. Only polyethylene ware was used in this work.

3. Results and discussion

Muscle parvalbumins are very suitable objects for fluorescence studies. Usually they contain nine to ten phenylalanine residues per molecule and lack both tryptophan and tyrosine residues. Some, however, contain one tyrosine and (or) one tryptophan residue per molecule [8]. The parvalbumin of whiting (*Gadus merlangus*) contains one tryptophan residue (Trp-102), one tyrosine residue (Tyr-26) and 10 phenylalanine residues [8]. The parvalbumin of pike (*Esox lucius*) p*I* 4.2 contains one tyrosine residue (Tyr-48) and 10 phenylalanine residues, while pike parvalbumin p*I* 5.0 contains only nine phenylalanine residues [12,13].

Parvalbumins are a family of low molecular mass proteins (11000–13000 Da). The three-dimensional structure of one of the carp parvalbumins shows that the molecule contains six helical regions [14]. Two pairs thereof (CD and EF) together with connecting loops form two high-affinity Ca^{2+}/Mg^{2+}-binding sites. The amino acid sequences of all known parvalbumins are homologuous. Therefore, one can assume that their tertiary structures are similar.

In our study we used Mg^{2+}-loaded parvalbumins instead of their Ca^{2+}-loaded forms, since thermal denaturation of Ca^{2+}-loaded parvalbumins occurs at temperatures which are too high (above 90°C) and thus not convenient for precise measurements.

α-Lactalbumin is also a low molecular mass (14200 Da) Ca^{2+}-binding protein [15,16] which contains four tryptophan, four tyrosine and four phenylalanine residues per molecule [17]. The primary structure of α-lactalbumin is very similar to that of lysozyme. It strongly binds one Ca^{2+} per molecule [16].

Fig. 1 demonstrates the temperature dependences of tryptophan fluorescence quantum yield, q, and spectrum position, $\bar{\lambda}$, for Mg^{2+}-loaded whiting parvalbumin (A) and Ca^{2+}-loaded α-lactalbumin (B). Heating induces a decrease in the tryptophan fluorescence yield and a very pronounced shift of the spectrum towards longer wavelengths. The red shift of the tryptophan fluorescence spectrum is undoubtedly caused by a thermal transition in the proteins structure. The

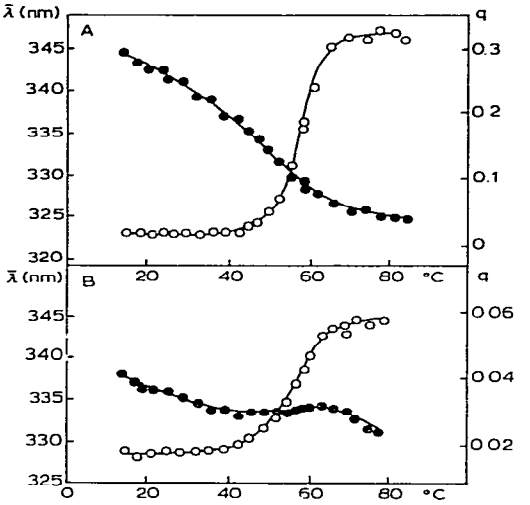

Fig. 1. Temperature dependence of tryptophan fluorescence spectrum position, $\bar{\lambda}$ (○), and quantum yield, q (●), for: (A) Mg^{2+}-loaded whiting parvalbumin (excitation wavelength 296.7 nm, protein concentration 0.024 mM, EGTA concentration 0.053 mM, Mg^{2+} concentration 3.3 mM); (B) Ca^{2+}-loaded bovine α-lactalbumin (excitation wavelength 296.7 nm, protein concentration 0.022 mM, Ca^{2+} concentration 0.33 mM). 10 mM Hepes, pH 8.1.

usual thermal quenching of tryptophan fluorescence complicates quantitative analysis of the temperature curve for the fluorescence yield or fluorescence intensity. The fluorescence intensity at a fixed wavelength $I_{\lambda,T}$ (or fluorescence quantum yield) for a given temperature T is:

$$I_{\lambda,T} = (1-\alpha) I^{N}_{\lambda,T} + \alpha I^{H}_{\lambda,T} \quad (1)$$

where the superscripts N and H refer to the native and 'high' temperature conformers, respectively, α is the fraction of conversion of the N to the H form, and $I^{N}_{\lambda,T}$ and $I^{H}_{\lambda,T}$ represent the intrinsic fluorescence intensity of the two conformers at the temperature of measurement. In order to determine α we have used two methods.

In the first method we have used fluorescence phase plots (the dependence of a fluorescence intensity at a fixed wavelength on a fluorescence intensity at another wavelength) [1]. Fig. 2 shows such phase plots for whiting parvalbumin (A) and α-lactalbumin (B). The linear parts of the plots

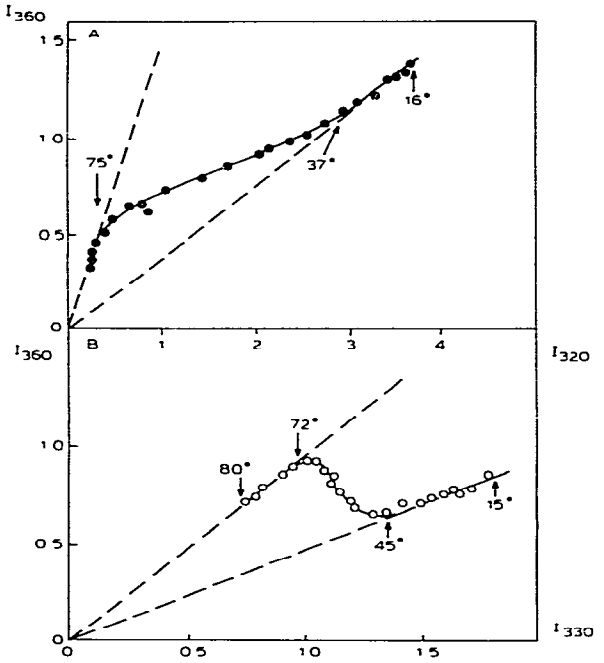

Fig. 2. Fluorescence phase plots for the temperature dependence of fluorescence of whiting parvalbumin (A) and bovine α-lactalbumin (B). Values of fluorescence intensities are expressed in relative units. Conditions as in fig. 1.

extrapolated to the origin correspond to the common thermal fluorescence quenching without any change in the protein structure. The complex curves between them correspond to thermal transitions in the protein structure. In the first method $I^{N}_{\lambda,T}$ and $I^{H}_{\lambda,T}$ were obtained by extrapolation of the linear parts of the phase plots to the thermal transition region. This method is useful only in the case when the thermal transition is accompanied by some changes in position or shape of the emission spectrum.

The second method of eliminating thermal quenching effects is based on the finding by Bushueva et al. [2,3] that the temperature dependence of the reciprocal fluorescence quantum yield (or reciprocal fluorescence intensity at a fixed wavelength) for native proteins containing a single fluorescing centre in the non-denaturing temperature range can be described by the equation:

$$1/q = a + bT/\eta \qquad (2)$$

where a and b are temperature-independent constants, T the temperature (K), and η the solvent viscosity (cP). They have assumed that the linearity of the plot of $1/q$ vs. T/η probably indicates that the mobility of internal parts in a protein globule is controlled by diffusion processes in the surrounding solvent. The main limitation of Bushueva's rule is the homogeneity of emitting centres in a sample in terms of eq. 2, since the presence of several kinds of fluorescent centres must lead to some non-linearity in the plot of the averaged $1/q$ value vs. T/η. Whiting parvalbumin chosen for this study is very suitable for the use of eq. 2, since it contains a single emitting tryptophan chromophore. Fig. 3 shows a plot of $1/q$ vs. T/η for whiting parvalbumin. The same figure demonstrates a plot of $1/q$ vs. T/η for α-lactalbumin. Despite the fact that α-lactalbumin possesses four fluorescent tryptophan residues, its plot $1/q$ vs. T/η, as in the case of the single tryptophan residue in whiting parvalbumin, has two linear parts corresponding to thermal quenching of the fluorescence of the native and heat-denatured protein. The curves between the linear parts correspond to

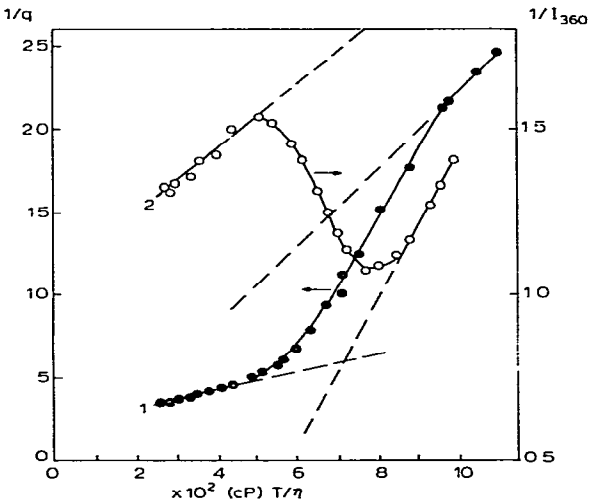

Fig. 3. Plots of $1/q$ vs. T/η for whiting parvalbumin (1) and $1/I_{360}$ vs. T/η for bovine α-lactalbumin (2). Conditions as in fig. 1.

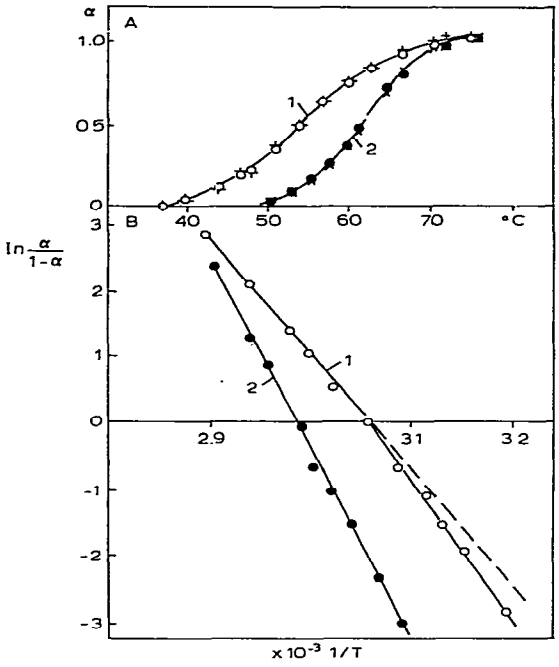

Fig. 4. (A) Temperature dependence of the parameter α (eq. 1) for the thermal transition in Mg^{2+}-loaded whiting parvalbumin (1) and Ca^{2+}-loaded bovine α-lactalbumin (2) obtained by two different methods (○ and +). (B) Van't Hoff plots for the thermal curves in A. (1) Whiting parvalbumin, (2) bovine α-lactalbumin. Conditions as in fig. 1.

the thermal transition in the protein structure. In the second method for elimination of thermal quenching effects, $I^{N}_{\lambda,T}$ and $I^{H}_{\lambda,T}$ or q^{N}_{T} and q^{H}_{T} were taken from an extrapolation of the linear parts of the plots of $1/q$ vs. T/η to the thermal transition region.

The temperature dependences of the parameter α obtained by the two methods for whiting parvalbumin and α-lactalbumin are shown in fig. 4A. One can see that the two methods described give practically the same results. Fig. 4B shows Van't Hoff plots of the thermal data. The plot is linear for α-lactalbumin but exhibits a break for whiting parvalbumin. This indicates the existence of at least two temperature-induced transitions in the parvalbumin. Table 1 lists thermodynamic parameters of the thermal transitions: transition enthalpy (ΔH), entropy (ΔS) and midpoint temperature (T_m). The parameters for parvalbumins were evaluated from the Van't Hoff treatment by fitting theoretical curves computed according to the scheme:

$$P_N \overset{K_1}{\rightleftharpoons} P_{H1} \overset{K_2}{\rightleftharpoons} P_{H2} \qquad (3)$$

(where P_N denotes native protein, P_{H1} and P_{H2} two different states of thermally changed protein, and K_1 and K_2 are the equilibrium constants at a given temperature T: $K_i = \exp[-(\Delta H_i - T\Delta S_i)/RT]$, $i = 1, 2$) to the experimental points.

The higher temperature step of the thermal transition for whiting parvalbumin is accompanied by an approx. 20 nm shift of its fluorescence spectrum to longer wavelengths. The spectral shift accompanying the lower temperature step is much smaller (≈ 2–3 nm), i.e., the process of exposure of the tryptophan residue to solvent occurs mostly in the higher temperature step of the thermal transition.

The second method of obtaining α can be used even in the case where the position or shape of an emission spectrum is not changed and the only informative parameter is fluorescence quantum

Table 1

Parameters of the thermal transitions in Ca^{2+}-loaded bovine α-lactalbumin and Mg^{2+}-loaded parvalbumins (PA) evaluated according to the Van't Hoff treatment of the denaturation curves in figs. 4 and 7

Protein	T_{m1} (°C)	ΔH_1 (kJ/mol)	ΔS_1 (J/K per mol)	T_{m2} (°C)	ΔH_2 (kJ/mol)	ΔS_2 (J/K per mol)
α-Lactalbumin	58	204 ± 20	610 ± 40			
Whiting PA	51	188 ± 40	577 ± 80	62	217 ± 40	644 ± 80
Pike PA pI 4.2	51	339 ± 40	1041 ± 100	64	401 ± 40	1191 ± 100
Pike PA pI 5.0	54	322 ± 40	991 ± 100	71	293 ± 40	853 ± 100

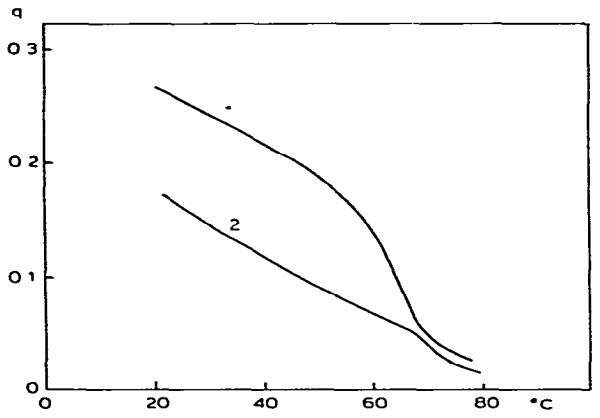

Fig. 5. Temperature dependence of quantum yield of tyrosine fluorescence of Mg^{2+}-loaded pike parvalbumin p*I* 4.2 (1) (excitation wavelength 280.4 nm, protein concentration 0.174 mM, EGTA concentration 6.2 mM, Mg^{2+} concentration 6.2 mM) and of phenylalanine fluorescence of Mg^{2+}-loaded pike parvalbumin p*I* 5.0 (2) (excitation wavelength 265. 2 nm, protein concentration 0.191 mM, EGTA concentration 6.0 mM, Mg^{2+} concentration 6.0 mM), 10 mM Hepes, pH 8.15.

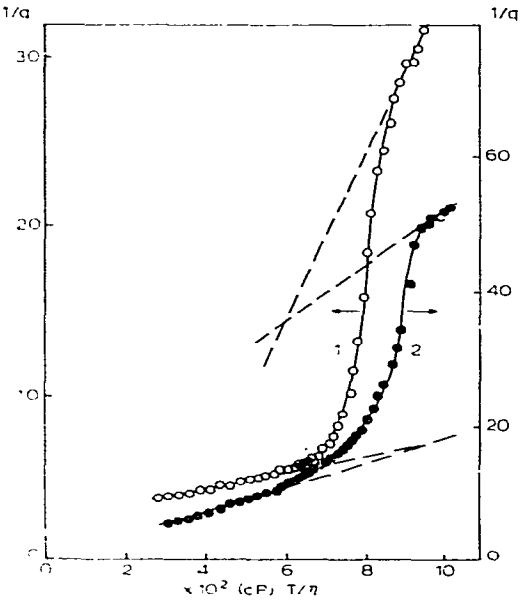

Fig. 6. Plots of $1/q$ vs. T/η for Mg^{2+}-loaded pike parvalbumins p*I* 4.2 (1) and p*I* 5.0 (2). Conditions as in fig. 5.

yield (or fluorescence intensity). This is the case for tyrosine and phenylalanine fluorescence of proteins.

Fig. 5 shows the temperature dependence of the tyrosine fluorescence quantum yield of pike parvalbumin p*I* 4.2 and that of the phenylalanine fluorescence yield of pike parvalbumin p*I* 5.0. The spectrum position for tyrosine and phenylalanine fluorescence remains constant in the temperature range 15–85°C. It can be readily observed in fig. 5 that it is very difficult to reveal the thermal transitions in pike parvalbumins from these temperature curves.

Pike parvalbumin p*I* 4.2 is suitable for the use of eq. 2, since it contains only one emitting tyrosine chromophore. Pike parvalbumin p*I* 5.0 contains nine phenylalanine chromophores, but due to the highly effective excitation energy migration between them [18], the system of phenylalanine residues behaves practically as a single emitter.

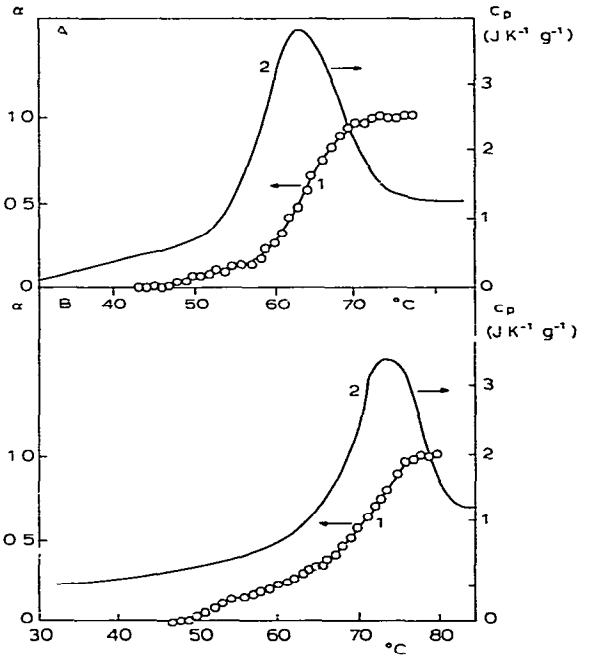

Fig. 7. Temperature dependence of the parameter α (1) (eq. 1) and of the partial specific heat capacity (2) for Mg^{2+}-loaded pike parvalbumins p*I* 4.2 (A) and p*I* 5.0 (B). Conditions as in fig. 5.

This is corroborated by the linearity of the $1/q$ vs. T/η plot for this parvalbumin in a rather wide temperature region. The plots of $1/q$ vs. T/η for the pike parvalbumins are presented in fig. 6. The curves between the two linear parts seem to correspond to temperature-induced transitions in the proteins. The temperature dependence of the parameter α for the pike parvalbumins, obtained by the method described above, is shown in fig. 7, in which can be seen the two-stepped character of the curves. The lower temperature steps of the curves are small, but well reproducible.

It is important to compare the data on fluorescence with those on microcalorimetry. Calorimetric recording made for Mg^{2+}-loaded pike parvalbumins under conditions similar to those of the fluorescence experiment are shown in fig. 7. The maxima of the asymmetric heat sorption peaks correlate with the mid-points of the higher temperature steps of the fluorescence curves. This also suggests that the main structural changes in parvalbumins occur in this step. The thermodynamic parameters of the thermal transitions in pike parvalbumins obtained by Van't Hoff treatment of the fluorescence data according to the scheme in eq. 3 are presented in table 1. The values of the transition enthalpy evaluated from the higher temperature steps of the fluorescence curves for pike parvalbumins pI 4.2 and 5.0 (401 $\pm$ 40 and 293 $\pm$ 40 kJ/mol, respectively) are in rather good agreement with the corresponding values of 382 $\pm$ 20 kJ/mol respectively obtained from the calorimetry data. Perhaps the calorimetric curves also contain two peaks, one of which is broad, located at lower temperatures and hardly revealed.

Thus, the methods described above allow one to obtain correct thermal transition curves free from the effects of common thermal quenching. These methods are suitable for tryptophan, tyrosine and phenylalanine fluorescence of proteins. Even the existence in a protein of several emitting centers is not a limitation on the use of these methods.

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