EQUILIBRIUM EVIDENCE OF NON-SINGLE STEP TRANSITION DURING GUANIDINE UNFOLDING OF APOMYOGLOBINS

C. BALESTRIERI, G. COLONNA, A. GIOVANE, G. IRACE and L. SERVILLO
Istituto di Chimica Biologica della I Facoltà di Medicina e Chirurgia-Università degli Studi di Napoli, Italy

Received 24 March 1976

1. Introduction

There are many experimental evidences that all myoglobins have the same basic structure [1–4]. It is generally assumed that the denaturation of these proteins is a single step process with variations in all physical properties which are closely related in occurrence and extent [3,5,6]. In this paper we report the effect of guanidine hydrochloride on intrinsic fluorescence and that of the 1-anilino-8-naphthalene sulfonate (ANS) conjugate of four different apomyoglobins, i.e. tuna, buffalo, beef, and sperm whale.

Tryptophan residues are located in the N-terminal branch of the globin molecule [7], whereas ANS is known to bind the apoprotein in the same non-polar moiety of the heme [8]. The N-terminal region is not involved in the formation of the heme pocket [9], therefore the environmental changes in the two molecular districts of the examined globins are independently evidenced by the variation of fluorescence behaviour observed in the two chromophores. The experimental data show an unexpected difference in sperm whale and tuna globin unfolding. The results are indicative that, during the course of denaturation, the conformational changes in the tryptophanyl segment are not thoroughly concomitant with those involving the heme pocket.

2. Materials and methods

Tuna, buffalo and beef myoglobins were prepared according to the method previously described [10]. Sperm whale myoglobin was purchased from Sigma Co. All proteins were used after a run on Sephadex G-75, equilibrated with phosphate buffer 5×10^{-2} M, pH 7.0. The homogeneity of the preparations was tested by disc gel electrophoresis at pH 8.6 on 7% polyacrylamide, as reported by Davis [11]. A single protein band was always observed by this technique. Apomyoglobins were prepared by butanone method of Teale [12]. In all cases no significant absorption due to heme was observed in the Soret region. The concentrations of apomyoglobins were determined by absorbance at 280 nm on a Zeiss PMQII spectrophotometer. The molar extinction at 280 nm of beef, buffalo and tuna were calculated from the tryptophan and tyrosine content [7,10], according to Wetlaufer [13]. The tryptophan and tyrosine content of tuna apoMb estimated by the method of Edelhoch [14] was 1 and 2 respectively. A value of 15 900 M^{-1} cm^{-1} was used for the molar extinction of sperm whale globin as reported by Harrison and Blout [15]. ANS was a product of Merck Co.; its Mg^{2+} salt was recrystallized twice with the method described by Weber and Young [16] and the molar extinction at 350 nm in 5×10^{-2} M phosphate buffer, pH 7.0, was observed to be $4.97 \pm 0.1 \times 10^{3}$ M^{-1} cm^{-1}. ANS apomyoglobins were prepared according to Stryer [8]. The denaturation equilibrium of apomyoglobins and ANS-apomyoglobin conjugates was measured by following the fluorescence intensities with an Hitachi Perkin-Elmer MPF2A recording spectrofluorometer equipped with thermostated ($20^{\circ}C \pm 0.2$) 1 cm quartz cuvet. The fluorescence measurements were made in the range where emission was linear with protein concentration. In denaturation experiments the protein was added

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to solutions of guanidine hydrochloride (Schwarz/Mann, Ultrapure), buffered with phosphate 5×10^{-2} M, pH 7.0.; 0.1 M KCl was present in all solutions. The fluorescence was then followed in time until an apparent equilibrium was reached. The reversibility of GuHCl denaturation at neutral pH was confirmed by diluting samples in concentrated denaturant with phosphate buffer.

3. Results and discussion

The guanidine hydrochloride unfolding of tuna, beef, buffalo and sperm whale apomyoglobin has been studied following the effect of increasing denaturant concentrations on the intrinsic protein fluorescence at neutral pH. In absence of denaturant the emission spectra of beef, buffalo and sperm whale apomyoglobin resulting from 288 nm excitation are very similar and the emission maxima are almost coincident (325 nm). The fluorescence emission maximum of buried tryptophan in proteins occurs at shorter wavelengths (320–330 nm) than tryptophan in aqueous solution (350–360 nm) [17]; the maxima centered at 325 nm indicate that all three proteins contain the two tryptophan residues in a relatively non-polar environment.

Tuna apomyoglobin contains a single tryptophan residue and its fluorescence emission maximum is at 320 nm, which is indicative of a minor access of solvent to indole ring.

The increase of guanidine hydrochloride concentration produces changes both in fluorescence magnitude and in the position of emission maximum. Since the variations of fluorescence intensity show a complex behaviour, we have followed the shift of the emission maximum as an index of the extent of protein denaturation [3]. The increase of guanidine hydrochloride concentration produces a red shift from 325 nm (320 nm for tuna globin) to about 345–350 nm. The relationships between the wavelengths of emission maximum of the examined apomyoglobins and the denaturant concentration is reported in fig.1. The experimental midpoints (defined as the concentration of denaturant required to effect 50% of the total change) are 0.95 M, 1.05 M, 1.15 M and 1.90 M respectively for buffalo, beef, tuna and sperm whale globin. The last value differs from the value of 1.6 M

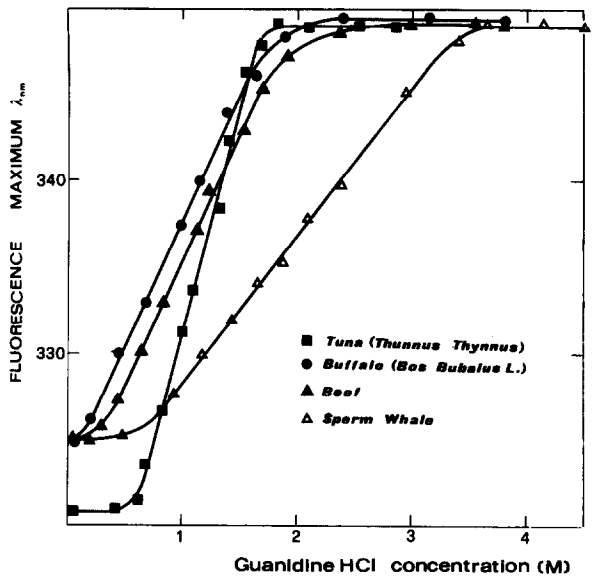

Fig.1. Wavelength of fluorescence emission maxima of apomyoglobins, with 288 nm excitation, as a function of guanidine hydrochloride concentration. All solutions contain 0.05 M phosphate (pH 7.0) and 0.1 M KCl. Protein concentrations varied between $0.5 \cdot 10^{-5}$ M and $0.9 \cdot 10^{-5}$ M. The fluorescence was followed with time until an apparent equilibrium was reached.

reported by Schechter and Epstein [3]. The results suggest a different conformational stability of these four apomyoglobins at neutral pH, the sperm whale globin seeming to be significatively more stable than those from the other species examined.

The position of fluorescence emission maximum provides information about changes occurring in the N-amino terminal region of the molecule which contains the two tryptophanyl residues in the examined mammalian apomyoglobins [7]. The N-terminal branch is not directly involved in forming the heme pocket [9]. In order to obtain information about the changes occurring in the heme district we have examined the effect of increasing denaturant concentrations on the fluorescence of the ANS bound to the globin.

The globins bind the ANS stoichiometrically in the same non-polar site of the heme in the molar ratio of 1:1 as reported by Stryer [8]. When the dye is bound to the globin, its quantum yield is 200-fold higher than in water with a concomitant blue shift

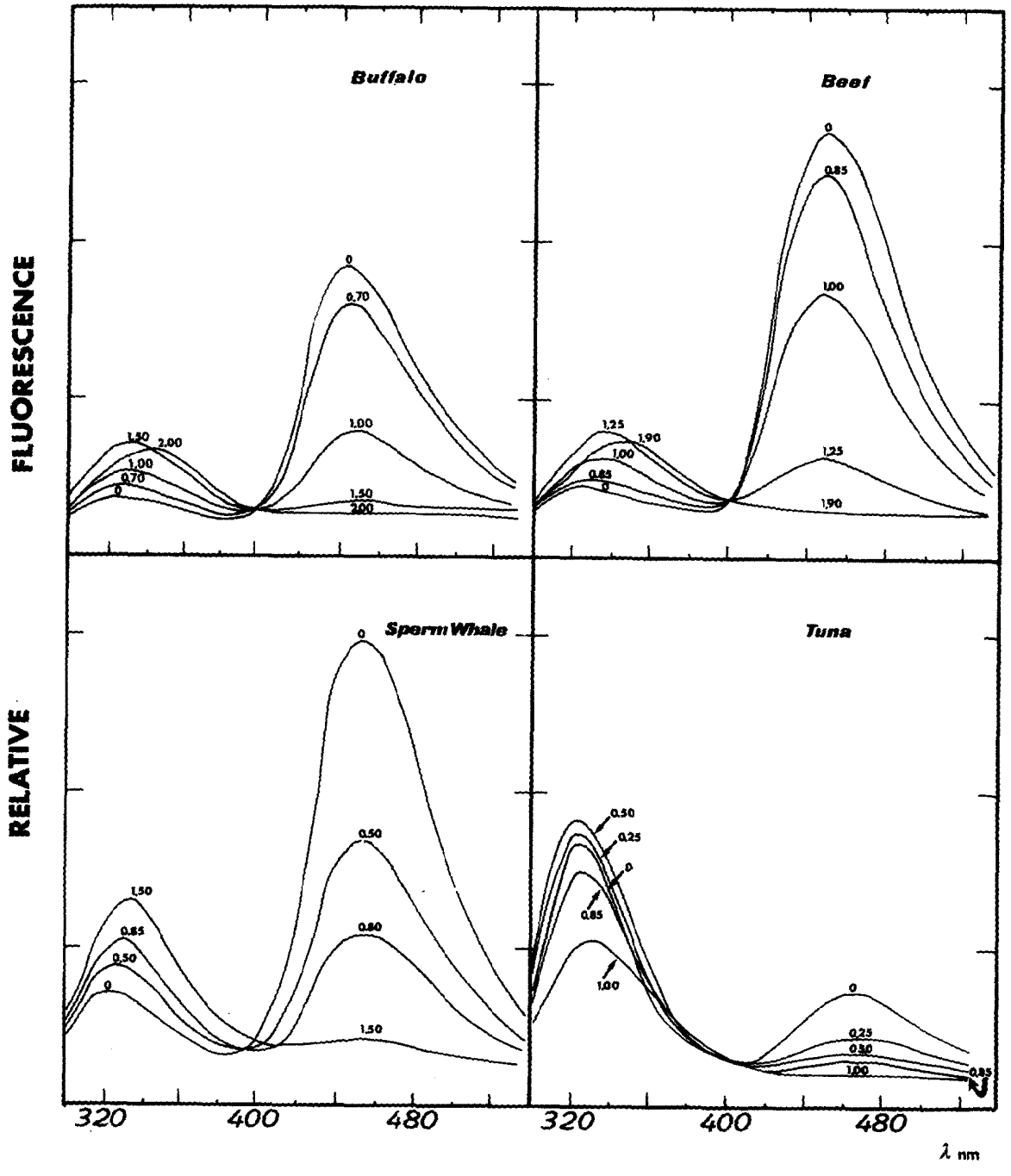

Fig. 2. Fluorescence emission spectra, resulting from 288 nm excitation, of ANS–globin conjugates at the indicated guanidine hydrochloride concentrations (molarity). Ordinates are fluorescence intensities in arbitrary units. All solutions contain 0.05 M phosphate (pH 7.0) and 0.1 M KCl. Protein concentrations were $0.7 \cdot 10^{-5}$ M.

of the emission maximum from 510 to about 450 nm. The occurrence of an energy transfer between tryptophan and the ANS molecule bound to the globin quenches the intensity of tryptophanyl emission when the ANS–globin conjugates are excited at 288 nm [8]. The fluorescence emission spectra obtained by excitation at 288 nm of ANS–globin conjugates in the wavelength region between 300 and 500 nm at neutral pH are reported in fig.2.

In absence of denaturant two well resolved maxima are evident in all spectra examined; the first one, located at 320–330 nm, reflects the remaining contribution of tryptophanyl emission; the second occurs at 450 nm and is characteristic of the ANS bound to the globin. The increase of denaturant concentration produces for buffalo, beef and sperm whale globin an enhancement of magnitude of tryptophanyl emission and a concomitant decrease of ANS fluorescence without any appreciable variation of the last emission maximum.

The efficiency of energy transfer between the single tryptophan residue of tuna globin and the bound ANS is lower than those observed in the other proteins examined. Between 0 and 0.5 M guanidine hydrochloride concentration there is an increase of tryptophanyl emission, whereas at higher denaturant concentration the fluorescence is strongly reduced. The ANS fluorescence of tuna ANS–globin conjugate decreases linearly with increasing concentration of denaturant. The dependence of the fluorescence intensity at 450 nm of the four examined ANS-globin conjugates from guanidine hydrochloride concentration is reported in fig.3.

Following the variation of ANS fluorescence at 450 nm sigmoidal curves are observed with transition midpoints of 0.25 M, 0.62 M, 0.95 M and 1.05 M respectively for tuna, sperm whale, buffalo and beef apomyoglobin. In the case of beef and buffalo globins there is an excellent agreement when the transition curves obtained following the two different fluorophores are compared. The transition midpoints of tuna and sperm whale globin obtained following the ANS fluorescence at 450 nm occur at lower denaturant concentrations then those measured by red shift of tryptophanyl emission. The observation that the emission maxima of all four ANS-globin conjugates remain constant at 450 nm suggest that the polarity of the microenvironment of the dye is

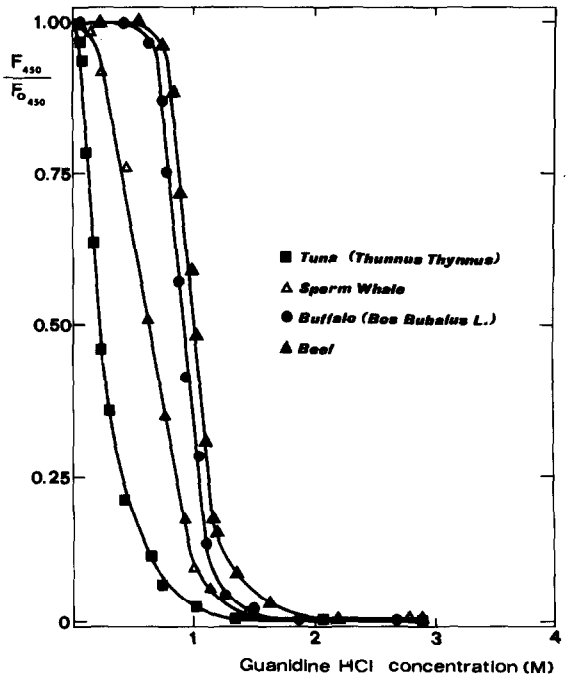

Fig.3. The dependence of fluorescence emission at 450 nm of ANS–globin conjugates from guanidine hydrochloride concentration. Ordinates indicate the ratio between the fluorescence intensity (F_{450}) at any guanidine hydrochloride concentrations and that observed in absence of denaturant ($F_{0\,450}$). All solutions contain 0.05 M phosphate (pH 7.0) and 0.1 M KCl. Protein concentrations were $0.7 \cdot 10^{-5}$ M. The fluorescence was followed with time until an apparent equilibrium was reached.

invariant during the course of the denaturation process; therefore the fluorescence variations at 450 nm only depend from the amount of protein molecules which retain the ability to bind ANS. The equilibrium data relative to tuna and sperm whale apomyoglobin indicate that a loosening of the tight folding of the native structure, leading to abolition of the capacity to bind ANS molecules, occurs as a separate process preceding the exposure of the tryptophan side chains to the solvent.

The denaturation of the myoglobins and their corresponding apoproteins is generally considered as a single-step process involving a cooperative transition between native and denatured form [3,5,6].

The parallelism of the transition curves of beef and buffalo apomyoglobin, obtained following the

two different fluorophores, indicates that the guanidine hydrochloride unfolding of these two proteins is a two-state process in the transition region [18,19]. It is also interesting to note that only the spectra of beef and buffalo ANS–globin conjugates passes through an isoemissive point at about 400 nm shared by the native and denatured form. The loss of isoemissive point of tuna and sperm whale ANS–globin conjugates at slightly higher denaturant concentrations than those corresponding to respective midpoints could indicate that the equilibrium mixture is not only composed by native and denatured protein, but appreciable concentrations of intermediate and structurally distinct form(s) of these globins could be present.

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