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Fluorescence study of the thyroxine-dependent conformational changes in human serum transthyretin

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Fluorescence studies of transthyretin (TTR) were conducted to detect structural changes associated with the environment of its two tryptophans, induced by binding of thyroxine (T_4). Non-radiative tryptophans relaxation rate has an activation energy of 6.4 kcal/mol for TTR, which is decreased to 4.4 kcal/mol for TTR-T_4 complex. The maximum fluorescence wavelength was red-shifted as the excitation wavelength was increased. T_4 changed the magnitude of this shift. T_4 binding per se changed the emission maximum reflecting different environments of the tryptophans. Double-quenching experiments also showed that T_4 produces changes in the tryptophans environments. These findings were interpreted as the result of structural alterations in the protein matrix induced by T_4 which contribute in part to explain the negative cooperativity associated with the occupancy of the second binding site.

Thyroxine binding; Human serum transthyretin; Tryptophan fluorescence; Conformational change

1. INTRODUCTION

Plasma transthyretin (TTR) is a homotetrameric protein of 55,000 molecular weight [1,2] with a physiological role in the transport of the hormone T_4 in man [3]. X-Ray crystallography [4,5] has established that two identical binding sites for T_4 are found in a central channel formed by the association of the four monomers. Two mol of T_4 are bound per mol of protein with binding constants two orders of magnitude different for each mol. This is interpreted in terms of a negative cooperativity between the sites [6,7]. The study described here was undertaken with the intention of using TTR Trp-41 and Trp-79 fluorescence which can give structural information, to detect conformational changes associated to its interaction with T_4.

2. MATERIALS AND METHODS

Human serum TTR was purified as described by Bashor et al. [8]. A single band was observed by SDS-gel electrophoresis at pH 8.9. Acrylamide and KI were Merck products. T_4 was obtained from Sigma. Fluorescence spectra and intensities were obtained with a Spex Fluorolog photon counting spectrofluorometer equipped with a thermostatted cell-holder. Tryptophan residues were selectively excited at 295 nm with a 5 nm bandwidth for excitation. Fluorescent quantum yields were estimated using neutral aqueous tryptophan solution as in [9]. TTR concentration was 1.5×10^{-5} M in 0.05 M Tris, 0.1 M NaCl, pH 8.6 and 20°C unless otherwise stated. T_4 concentration was 2.6×10^{-5} M making a bound-T_4/TTR mol ratio of 1.7, ensuring thus the operation of negative cooperativity between the two binding sites. Double-quenching experiments were performed according to Somogyi et al. [10]. The inner filter effect was reduced with front face illumination to minimize the absorptive screening.

Abbreviations: TTR, transthyretin; T_4, L-thyroxine.

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3. RESULTS

3.1. *Internal quenching–temperature effect*

Changes in quantum yield of protein fluorescence may be obtained not only by the effect of external quenchers but also by groups of atoms in the protein capable of quenching the fluorescence of tryptophan groups in close proximity. This type of quenching is limited by the frequency of active collisions of excited tryptophans with the near group quenchers. The curves of thermal quenching of emission indicate the temperature dependence of the frequency of active collisions and therefore the dynamic mobility rate of protein structures surrounding the tryptophans [11]. Their non-radiative de-excitation rate is limited by the activation energy of quenching, E_a, which can be calculated from the slope of the straight line obtained by plotting log $(1/Q - 1)$ versus $1/T$, where Q is the fluorescence quantum yield of tryptophans and T is the absolute temperature [12].

Since TTR tryptophans are non-uniform in their environments the protein virtually presents a multicomponent system whose emission yield may be described only by averaged characteristics. From Fig. 1 we have obtained E_a values of 6.4 ± 0.3 and 4.4 ± 0.2 kcal/mol for TTR and TTR–T_4 complex, respectively. The ob-

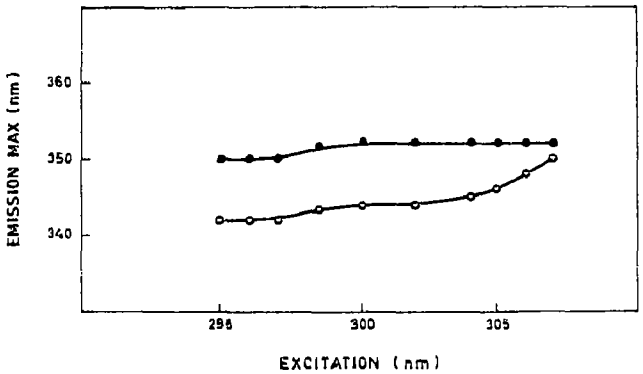

Fig. 1. Temperature dependence of tryptophan fluorescence quantum yield (Q) for TTR (○) and T_4–TTR complex (1.7 mol T_4/mol TTR) (●). The wavelengths of excitation and emission were 295 and 342 nm, respectively.

served values show that on average T_4 interaction produces a change in the environments of the TTR tryptophans.

3.2. *Red-edge excitation*

If fluorescence is excited by a light quantum whose energy is lower than the average energy of the tryptophans electronic transition, there will occur a selective excitation within the ensemble. This may shift the fluorescence spectra toward the long wavelength region [13]. Fig. 2 illustrates the dependence of the fluorescence spectra on the excitation wavelength for TTR and TTR–T_4 complex. TTR emission shows a progressive shift toward longer wavelengths with increase in excitation wavelength. This observation shows the heterogeneity of environments of the two tryptophans residues and also that preferential excitation is possible. The interaction of T_4 makes less significant the long wavelength shift. This can be interpreted as that the two tryptophans have now become more or less equivalent or at least have sufficient mobility to become equivalent during the lifetime of their excited states. These results

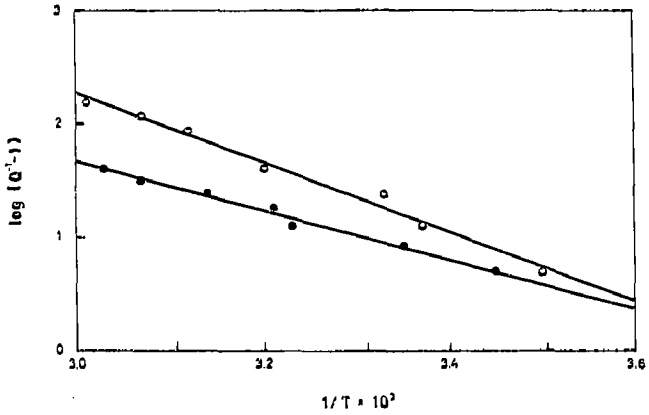

Fig. 2. Dependence of maximum wavelength of the fluorescence spectra on the excitation wavelength for TTR (○) and T_4–TTR complex (1.7 mol T_4/mol TTR) (●).

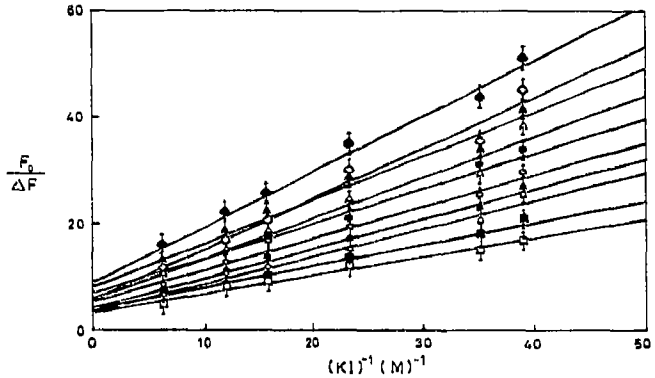

Fig. 3. Quenching of TTR by iodide in the presence of varying amounts of acrylamide. The acrylamide concentrations were as follows: 0 (□), 0.1 (○), 0.2 (▽), 0.3 (△) and 0.4 M (◇). Full symbols represent quenching of T_4–TTR complex (1.7 mol T_4/mol TTR). The wavelengths of excitation and emission were 295 and 342 nm, respectively.

show that a change in the environment of tryptophans is occurring as result of T_4 interaction with TTR.

3.3. *T_4 quenching*

Fluorescence quenching of TTR tryptophans by T_4 has been described by Nilson and Peterson [14] in a study of its binding properties. We have found that the extent of quenching by T_4 increases when it is measured at lower emission wavelengths. At a T_4 content of 1.7 mol per mol of TTR values of 40% at 350 nm, 51% at 340 nm and 65% at 325 nm were observed. This can be interpreted as that the emission spectrum is composed of two overlapping spectra and that the individual emissions are quenched to different degrees by T_4. The same effect can also be observed by considering that a shift in the emission peak from 342 nm to 350 nm is observed with T_4 binding (not shown but see Fig. 2 at 295 nm excitation wavelength), showing that the tryptophan emitting at lower wavelengths is more strongly quenched. This should be in an environment more excluded from contact with solvent molecules and should correspond to Trp-79.

These results can be interpreted in terms of conformational origin; however, they can also be explained as an

Table 1

Quenching constants (K) (M^{-1}) and tryptophans exposed to (external) fluorescence fraction (a) and buried (internal) fluorescence fraction (b) for transthyretin fluorescence quenching

Quencher		Transthyretin	Transthyretin + Thyroxine
Iodide	K_2	7.9 ± 0.4	6.7 ± 0.3
	a	0.40 ± 0.02	0.32 ± 0.01
Acrylamide	K_{1e}	1.8 ± 0.1	2.8 ± 0.1
	K_{1i}	3.8 ± 0.1	3.4 ± 0.1
	b	0.52 ± 0.02	0.45 ± 0.02

energy transfer process. Trp-79 is relatively near the binding site, in fact a minimal contact of 13.6 Å is found with one of the T_4 iodines (13′), and the critical transfer distance (R_o) of the Förster theory for transfer from tryptophan to ionized T_4 has been calculated to be 23.3 Å [15].

3.4. *External double-quenching*

Depending on their location in the protein matrix tryptophan residues may be fully or partially exposed to the solvent. Ionic quenchers can extinguish only fluorescence emitted by tryptophans located at or near to the surface of the protein [16]. In contrast, non-ionic quenchers can penetrate the protein matrix and quench all the tryptophan residues, exposed and buried [17]. By means of a double-quenching method which requires the simultaneous application of these two types of quenchers it is possible to achieve a separate analysis of the quenching parameters characteristic of the solvent exposed and solvent-masked tryptophans [10].

The method was applied to resolve TTR and TTR–T_4 complex fluorescence by the use of iodide as ionic or selective and acrylamide as non-ionic or non-selective quenchers. The plot shown in Fig. 3 is a set of data from quenching experiments carried out by using iodide to titrate samples of TTR and TTR–T_4 at different concentrations of acrylamide. From the slopes of the curves a secondary plot is obtained for the evaluation of the quenching constants as described by Somogyi et al. [10]. Table I shows that T_4 decreases the exposed tryptophan quenching constant K_2 for iodide. This is a consequence of a decreased accessibility of the tryptophan to the solvent due to a probable association with a hydrophobic environment. This is strengthened by the observation of a diminished fraction of the exposed (external) tryptophan fluorescence (a) upon T_4 interaction. Changes in K_{1e} and K_{1i} or acrylamide quenching constants for external and internal tryptophans, respectively, cannot be easily interpreted because acrylamide is quenching both simultaneously. However, the observed diminished fraction of the buried (internal) tryptophan fluorescence (b) after T_4 binding suggests an increased association of the buried tryptophan with a more external or hydrophylic environment.

4. DISCUSSION

TTR contains two tryptophans per subunit located in different environments, Trp-41 is in a solvent exposed area and positioned between Lys-35 and Lys-70 whereas Trp-79 is completely buried and close to Ile-84, Leu-111 and Pro-113. The different tryptophan locations were correctly sensed by quenching and red-edge excitation experiments. T_4 interaction with TTR under different conditions produces changes in the tryptophan emission profiles which can be interpreted as a result of structural modifications in the protein which make Trp-41 environment less hydrophylic and Trp-79 less hydrophobic. What is probably occurring is a diminished interaction between Trp-41 and the two lysines together with an improved interaction with the near Phe-33 which is perpendicularly-oriented with Trp-41 and at a distance of 7.1 Å. Trp-79 is nearer to the T_4 binding site than Trp-41. It is at 13.6 Å from one of the 3′ iodines of T_4. Since the hydrophilic residues Ser-115, Ser-117 and Thr-119 with their associated water molecules form a complex hydrogen-bonded network at the centre of the binding channel, and the effect of hormone binding results in an altered pattern of hydrogen bonding in this area [18], it is probable that this alteration may be propagated to become the cause of the increased polarity in the Trp-79 environment.

Negative cooperativity of T_4 binding can arise from an interaction between the bound ligands by steric effect, or from an interaction between the two sites mediated by conformational changes in the protein. A steric interaction has been excluded by X-ray diffraction studies [4] which clearly show that the hormones bind at two discrete and non-overlapping sites within the TTR channel, separated by a distance of 8 Å. Ultracentrifugation [14], circular dichroism [19] and X-ray [9] studies have shown no substantial changes in the conformation of TTR on hormone binding. This has prompted suggestions that the negative cooperativity may be transmitted by a change in the network of hydrogen bonds linking the two non-contiguous sites. Our fluorescence results reveal internal dynamic changes in the protein induced by the hormone binding which must accompany the alterations in the hydrogen bonding network and they should also contribute to inducing motional restrictions in the protein, making the second binding site no longer flexible so that the second hormone molecule cannot enter with the same ease as the first.

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