

## UNMASKING OF TYROSYL FLUORESCENCE IN SERUM ALBUMINS ON BILIRUBIN BINDING

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### 1. Introduction

The intrinsic fluorescence of proteins arises principally from the aromatic amino acids tyrosine and tryptophan. Fluorescence of tryptophan predominates even in proteins containing much more tyrosine than tryptophan, for a variety of reasons including the relatively high absorbance of tryptophanyl residues, low fluorescence quantum yields of most tyrosyl residues and the existence of efficient mechanisms for the transfer of excitation energy from tyrosyl to tryptophanyl residues [1]. Even HSA, which contains 1 tryptophanyl and 18 tyrosyl residues, displays fluorescence characteristic of tryptophan and a sophisticated mathematical analysis was required to demonstrate the tyrosyl contribution [2].

Serum albumins are known to bind a variety of ligands with high affinity [3] and serve as carriers for many of them. It has been proposed that they possess 'configurational adaptability' that accounts for conformational changes on ligand interaction, co-operative binding effects and the varied structures of acceptable ligands [4]. HSA contains 1 Trp and BSA, 2 Trp, while both contain 18 Tyr residues. There has been no detailed study of the tyrosyl fluorescence as it is masked by the tryptophanyl fluorescence. Bilirubin is known to bind strongly to serum albumins [5] and to quench their tryptophanyl fluorescence by energy transfer from Trp to bilirubin [6]. We here report that this quenching of tryptophanyl fluorescence unmasks the tyrosyl fluorescence. The unmasking is pronounced for HSA and BSA but is not observed for many pro-

teins that do not have a high affinity for bilirubin. Studies on the exposure of tyrosyl residues as probed by iodide quenching indicate that the residues in HSA are less accessible to quencher than those in BSA.

### 2. Materials and methods

HSA, BSA (both essentially fatty acid free), pepsin, lysozyme, ovalbumin,  $\alpha$ -chymotrypsin, Tris, NATA and bilirubin were from Sigma Chemical Co. All other chemicals were of analytical grade. Fluorescence measurements were carried out on a Perkin Elmer Model MPF-44A fluorescence spectrophotometer, operated in the ratio mode, with 5 nm excitation and emission band pass. All measurements were carried out in 10 mM Tris-HCl (pH 8.0) except pH variation experiments which were carried out in unbuffered solutions.

### 3. Results

The fluorescence spectra of human and bovine serum albumins excited at 275 nm in the presence and absence of 1.3 equiv. bilirubin are shown in fig.1. It can be seen that the tryptophanyl fluorescence is specifically quenched, unmasking the tyrosyl fluorescence at 305 nm. At the bilirubin concentrations used the protein binding site is saturated and the 1:1 complex is observed [5,7]. The spectrum in fig.1A suggests that bilirubin binding almost completely quenches the lone Trp residue (Trp 214) [8] on HSA. However, a shoulder is seen at 335 nm in the BSA-bilirubin spectrum (fig.1B), in agreement with the report [6] of 80% quenching of tryptophanyl fluorescence by bilirubin. This is presumably due to Trp 134 on BSA

**Abbreviations:** HSA, human serum albumin; BSA, bovine serum albumin; Tris, tris (hydroxymethyl) amino methane; NATA, *N*-acetyltyrosinamide; *F*, fluorescence intensity (arbitrary units)

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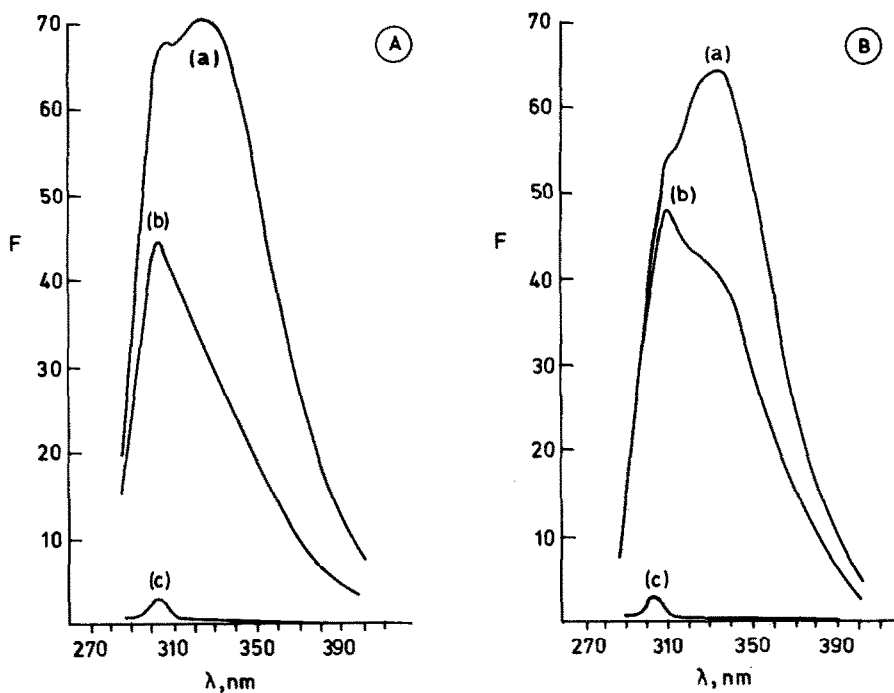


Fig.1. Fluorescence emission spectra of serum albumins,  $\lambda_{ex} = 275$  nm: (A) a, HSA; b, HSA + 1.3 equiv. bilirubin; c, Raman band of water. (B) a, BSA; b, BSA + 1.3 equiv. bilirubin; c, Raman band of water.

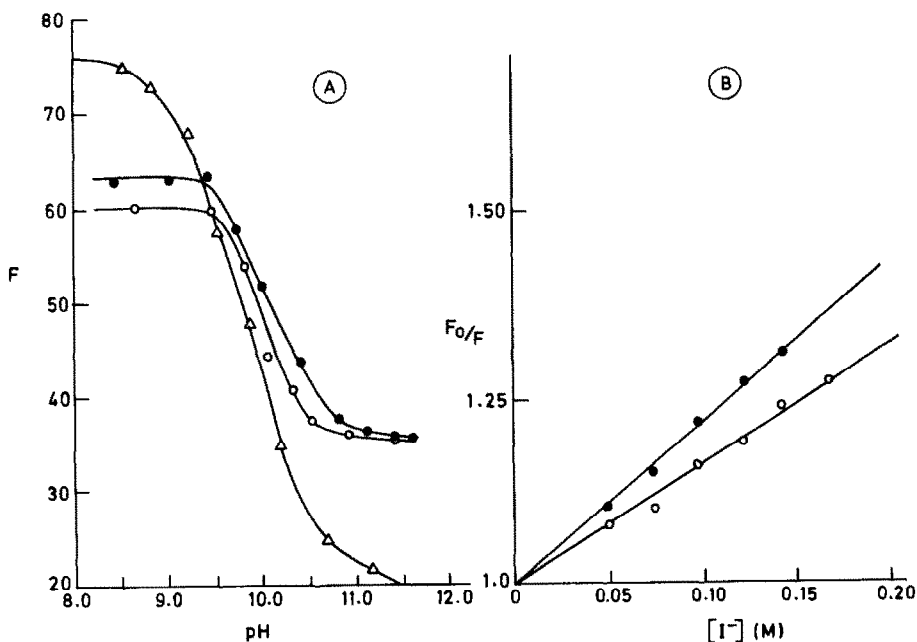


Fig.2. (A) pH variation of 305 nm emission peak,  $\lambda_{ex} = 275$  nm: (●—●) BSA-bilirubin; (○—○) HSA-bilirubin; (△—△) NATA. (B) Stern-Volmer plots of iodide quenching of serum albumin-bilirubin complexes: (●—●) BSA-bilirubin; (○—○) HSA-bilirubin.

being less quenched than Trp 212. Comparison of the sequences of HSA and BSA shows that Trp 214 on HSA corresponds to Trp 212 on BSA [8]. The quenching in BSA has been ascribed to energy transfer from tryptophan to bilirubin with an average Trp–bilirubin distance estimated as 25 Å [6]. It is possible that the bulk of the transfer observed is from Trp 212 which may be located closer to the bilirubin binding site, or in a more favourable orientation, than Trp 134, although both have been suggested to be present in the binding cleft for bilirubin [9]. Earlier studies of Trp quenching by bilirubin in serum albumins were based on excitation at 290 nm [6] which would be unfavourable for the detection of tyrosyl fluorescence.

The pH dependence of the 305 nm peak in the protein–bilirubin complexes and NATA is shown in fig.2A. The  $pK_a$  values obtained from the titration curves are  $\sim 9.9$  for the complexes while that for NATA is  $\sim 9.7$ . This supports the assignment of the emission peak at 305 nm in the protein–bilirubin complexes to protein tyrosyl residues.

A study of the average accessibility of the tyrosyl

residues as probed by iodide quenching [10] yielded linear Stern-Volmer plots over 0.05–0.15 M iodide with quenching constants of  $2.1 \text{ M}^{-1}$  for BSA and  $1.6 \text{ M}^{-1}$  for HSA (fig.2B). This suggests that despite similarities in sequence the tyrosyl residues of BSA are more exposed to quenchers than those of HSA. Further support for possible structural differences between the serum albumins comes from studies of the acrylamide quenching of the dansyl fluorescence of serum albumins dansylated at Cys 34. These studies indicate that the sulfhydryl in HSA is more accessible to quencher than that in BSA (M. K. M., P. B. unpublished).

Attempts were made to unmask the tyrosyl fluorescence of ovalbumin, pepsin, lysozyme,  $\alpha$ -chymotrypsin and trypsin. Fig.3A shows the effect on the ovalbumin emission spectrum of 5 equiv. bilirubin. Aside from a general decrease in fluorescence intensity (accounted for by the  $A_{275}$  of bilirubin) no selective quenching is seen. Similar results were obtained with lysozyme, pepsin and  $\alpha$ -chymotrypsin. Some changes in band shape (not accounted for by the Raman band of

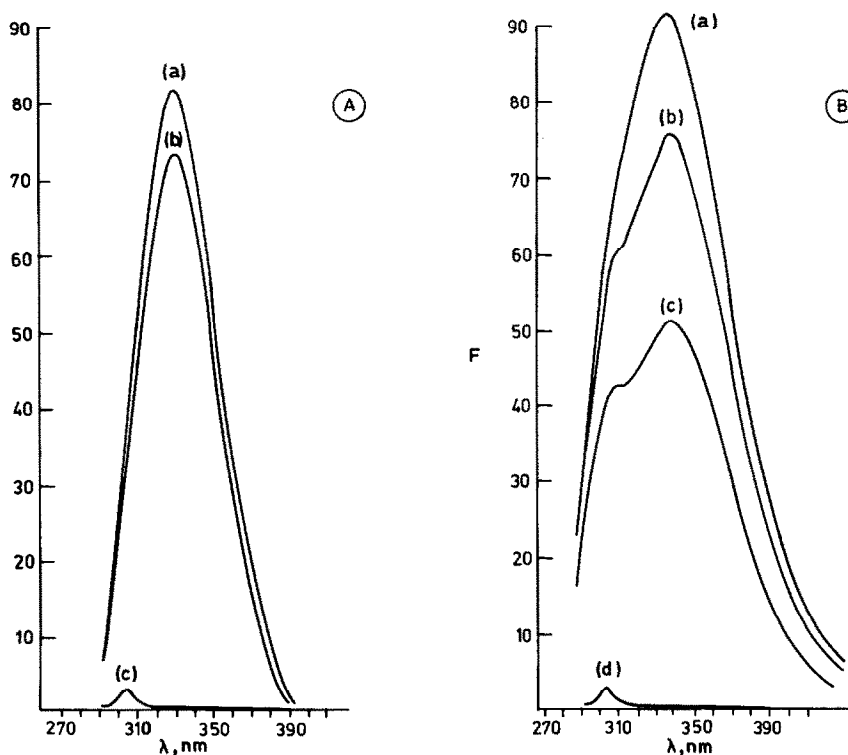


Fig.3. Fluorescence emission spectra of proteins  $\lambda_{\text{ex}} = 275 \text{ nm}$ : (A) a, ovalbumin; b, ovalbumin + 5 equiv. bilirubin; c, Raman band of water. (B) a, trypsin; b, trypsin + 5 equiv. bilirubin; c, trypsin + 10 equiv. bilirubin; d, Raman band of water.

water) suggestive of a lowered Trp contribution is observed in the case of trypsin at 5 and 10 equiv. bilirubin (fig.3B). It may be noted that HSA tyrosines are completely unmasked even at 1.3 equiv. bilirubin.

The unmasking of tyrosyl fluorescence by bilirubin appears to be specific for serum albumins and provides a spectroscopic handle for the study of the tyrosyl environments in these proteins.

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