Protein–Peptide Interactions as Probed by Tryptophan Fluorescence: Serum Albumins and Enkephalin Metabolites

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Binding of Leu-enkephalin and the enkephalin metabolite, tyrosine-glycine-glycine (TGG), to bovine serum albumin (BSA) was studied as a model to investigate protein peptide interactions. TGG and Leu-enkephalin quench the tryptophyl fluorescence of BSA. Stern-Volmer quenching constants were typically in the range of 40 to 300 M^{-1}, depending on the experimental conditions. The addition of Cu(II) or Ni(II) did not change the quenching constant, indicating that TGG does not compete for the metal binding sites on BSA. From fluorescence quenching studies with TGG, tyrosyl-glycine, tyrosine and glycyl-glycine, it was concluded that the presence of the tyrosine residue is required for the observed quenching. The phenolic group in tyrosine accounted for the quenching mechanism because phenol was efficient in quenching BSA fluorescence, whereas phenylalanine had no detectable effect. A large solvent isotope effect on the quenching constant of phenol and TGG with BSA strongly suggests an active role of the $-OH$ functionality in the quenching mechanism.

KEY WORDS: fluorescence; quenching; peptide; protein; isotope effect; tryptophan; tyrosine; Leu-enkephalin.

INTRODUCTION

The rational design of a drug delivery system demands a thorough understanding of the intermolecular interactions between the drug and the macromolecular carrier. This prompted us to study the binding of enkephalin and related peptides to human and bovine serum albumins (HSA and BSA) by fluorescence spectroscopy. The intrinsic fluorescence of the Trp residues at positions 212 and 134 in BSA (1) provides a convenient and sensitive spectroscopic means to study its interaction with peptides. The tripeptide, Tyr-Gly-Gly (TGG), representing the first three residues of enkephalins and endorphins, was chosen as a model peptide. The use of small fragments of a larger peptide may allow one to study their contribution to the overall interaction with the target protein. Although TGG itself does not show significant opioid activity, it is one of the metabolites of enkephalin (2).

MATERIALS AND METHODS

Crystallized and lyophilized bovine serum albumin, 98–99%, human serum albumin (fatty acid free), Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu), Tyr-Gly-Gly (TGG), Tyr-Gly, Gly-Gly, and Tyr were obtained from Sigma Chemical Co. Tris, phenol, and D_2O (reagent grade) were obtained from Aldrich Chemical Co. All solutions were prepared in double-distilled water and studies were carried out in 0.2 *M* Tris buffer at pH 8.5. The pH of the solutions was adjusted at 25°C using an Orion 901 pH meter.

Quenching Experiments. Steady-state fluorescence spectra were recorded on a Perkin-Elmer LS-5B luminescence spectrometer, interfaced to an Apple Macintosh computer. Data were collected and analysed by software developed in our laboratory. Samples were excited at 295 nm as indicated to minimize contributions from tyrosine fluorescence. Fluorescence intensities of albumin (75 μ*M*) at 342 nm were measured at various TGG concentrations (0.5–7.5 m*M*). Alternatively, the area under each spectrum was calculated and used in the quenching measurements. Quenching constants obtained from single-wavelength intensity measurements were compared with results from area measurements to check for consistency. Both sets of data agreed within ~6%. In order to minimize the scattered light, the excitation and the emission slit widths were adjusted to 3 and 5 nm, respectively. Similar experimental conditions were used in all the fluorescence quenching studies with Leu-enkephalin, Tyr-Gly, Gly-Gly, tyrosine, and phenol.

RESULTS AND DISCUSSION

Bovine serum albumin consists of 582 amino acids, with one Trp residue at 212 in the interior and an additional Trp residue located at 134, closer to the protein surface. Human serum albumin, on the other hand, consists of 585 amino acids, with only one Trp located at the 214th residue (1). Excitation at the absorption bands of the Trp residue at 295 nm results in a strong fluorescence emission characteristic of this Trp residue, with a maximum around 340 nm. The excitation wavelength (295 nm) was chosen to minimize tyrosyl fluorescence. Excitation of BSA and HSA solutions at 295 nm resulted in emission spectra with maxima at 342 and 340 nm, respectively, as observed previously (3,4).

The interaction of the tripeptide, TGG, with albumins can be monitored by fluorescence quenching experiments. Albumin fluorescence is quenched by TGG at millimolar concentrations. The Stern-Volmer quenching constant (5), K_{sv}, was obtained from steady-state fluorescence intensity measurements and by integrating the spectra at various quencher concentrations. The following equation was used to estimate the K_{sv} values:

$$\frac{A_0}{A} \quad \text{or} \quad \frac{I_0}{I} = 1 + K_{sv}[Q] \tag{1}$$

where I and I_0 are the intensities and A and A_0 are the areas under the curve, in the presence and absence of the quencher, Q, respectively. Quenching plots for BSA and HSA at various concentrations of TGG are shown in Fig. 1. Even at the highest concentration of TGG employed, no light was absorbed by the quencher, ensuring that the quenching was not due to an internal filter effect. Therefore, the ob-

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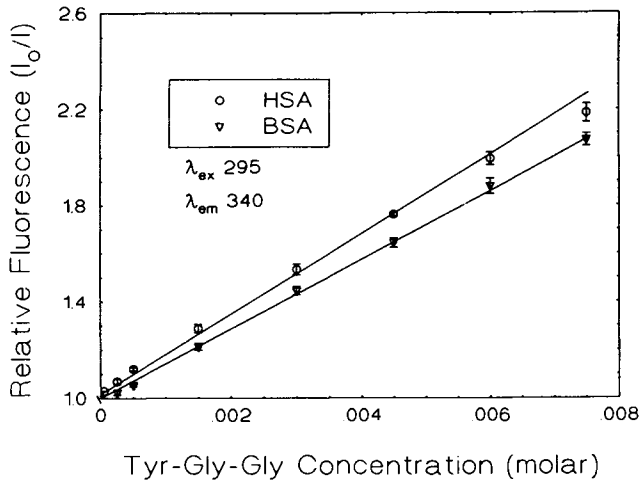

Fig. 1. Quenching plots (I_o/I versus Q) for BSA and HSA in the presence of Tyr-Gly-Gly at 295 nm excitation in 0.2 M Tris buffer at pH 8.5.

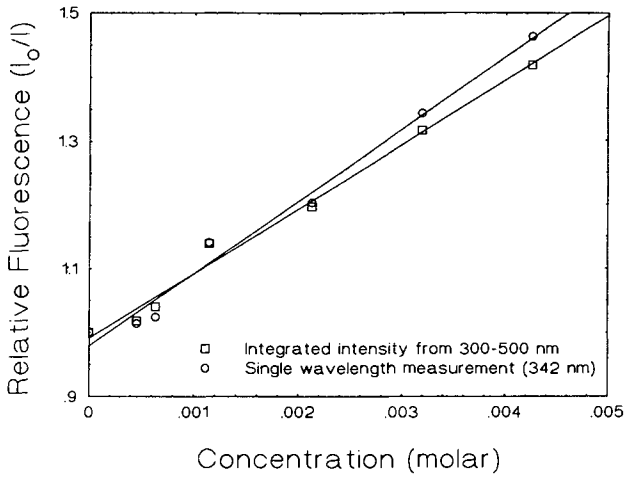

Fig. 2. Single wavelength and integrated intensity quenching plots (I_o/I versus Q) for BSA in the presence of Leu-enkephalin upon excitation at 295 nm in 0.2 M Tris buffer at pH 8.5.

served quenching can be interpreted in terms of a quenching mechanism involving TGG and serum albumin interactions. A quenching constant of 146 ± 2 M^{-1} was estimated from the intensity measurements with BSA when TGG was used as the quencher. Similar results were obtained from the integrated area measurements, with a deviation of ±6%.

In order to determine the role of the surface Trp residue in BSA in the quenching mechanism, experiments were carried out with HSA, which has only the interior Trp residue in loop 4. The surface residue in BSA is expected to be more accessible to the quencher than the interior Trp residue, and therefore, a lower quenching constant is expected with HSA due to the absence of the surface residue. However, the observed quenching constant with HSA (160 ± 3 M^{-1}) was greater than with BSA (145 ± 3 M^{-1}). Hence, the fluorescence from the interior residue in HSA is more efficiently quenched than that of the two Trp residues present in BSA. The difference in quenching efficiency may be due to various factors such as fluorescence lifetimes, internal energy redistribution, and the access to the external quencher.

The interaction of Leu-enkephalin with BSA was subsequently investigated. BSA fluorescence is quenched by Leu-enkephalin, and the resulting Stern-Volmer curves from the intensity and area measurements are shown in Fig. 2. The plots are linear, with slopes of 113 ± 3 and 101 ± 3 M^{-1} for the intensity and area measurements, respectively. The two slopes are in close agreement, indicating the validity of the intensity measurements. The slopes observed with Leu-enkephalin, however, are slightly smaller than the estimates obtained with TGG. These experiments indicate that TGG and Leu-enkephalin have similar binding interactions with albumin.

Since metal ions such as Cu^{2+} and Ni^{2+} have been shown to bind to BSA at the amino terminus (6), the effect of these ions on TGG quenching was investigated to determine if TGG competes for metal binding sites or if metal ions can promote TGG quenching efficiency. The quenching constants for TGG with BSA in the presence of Cu^{2+} and Ni^{2+} did not change significantly. Thus, quenching by TGG is not affected by the presence of these metal ions. Therefore, the site of interaction of TGG is not the same as the metal binding site.

In order to establish the role of tyrosine chromophore in the quenching mechanism, quenching by Gly-Gly, Tyr-Gly, and Tyr was investigated. The observed quenching constants strongly depended on the presence of tyrosine residue in the quencher. The estimated K_{sv} values with these quenchers are 0 ± 1, 120 ± 1, and 295 ± 7 M^{-1} for Gly-Gly, Tyr-Gly, and Tyr, respectively. Since the dipeptide Gly-Gly does not quench the emission, the tyrosyl group in the quencher is essential for the quenching process. Consistent with this suggestion, Tyr itself quenches the emission more efficiently than Tyr-Gly or Tyr-Gly-Gly.

Several mechanisms can be suggested to explain the quenching results. The singlet excited-state energy for the Tyr residue is higher than that of Trp (7). Therefore, direct energy transfer from Trp singlet excited state to Tyr is highly unlikely. Electron and proton transfer mechanisms provide two alternative pathways for the deactivation of the Trp excited state by Tyr.

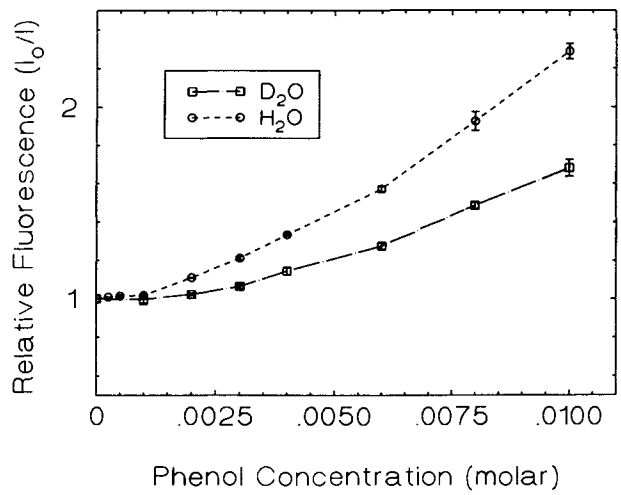

Fig. 3. Solvent isotope effect on the fluorescence quenching of BSA by phenol upon excitation at 295 nm in 0.2 M Tris buffer at pH 8.5.

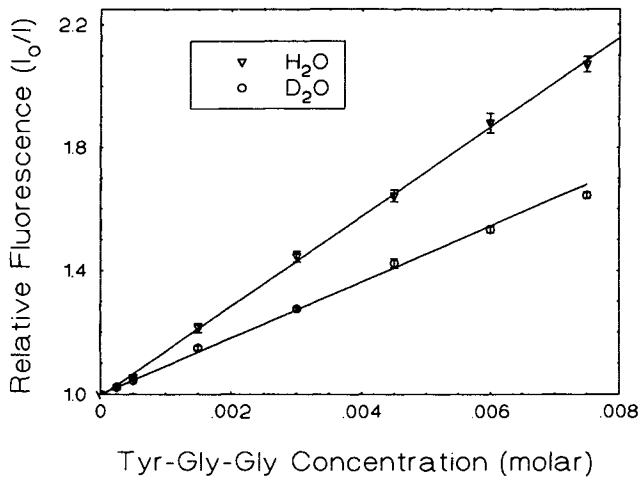

Fig. 4. Solvent isotope effect on the fluorescence quenching of BSA by Tyr-Gly-Gly upon excitation at 295 nm in 0.2 *M* Tris buffer at pH 8.5.

Since Tyr residue is necessary for the quenching process, the roles of the aromatic ring and the phenolic hydroxy group present in Tyr were explored for their fluorescence quenching ability by using phenylalanine and phenol as fluorescence quenchers. Phenylalanine did not quench BSA fluorescence, whereas phenol quenches BSA fluorescence efficiently. Thus, the phenolic side chain but not the aromatic ring is crucial for the quenching behavior. In order to determine the role of proton corresponding to the phenolic side chain in the quenching mechanism, experiments were carried out in H_2O and D_2O. A large isotope effect on the quenching constants would indicate the participation of the hydroxy function in the quenching mechanism. The quenching data with phenol and BSA in D_2O and H_2O are shown in Fig. 3. A strong isotope effect is clearly evident from the data and confirms the participation of the $-OH$ function in the quenching mechanism. Experiments with TGG and BSA were also carried out in D_2O to test if TGG quenching takes place by a similar mechanism (Fig. 4). The quenching constant decreased from 146 ± 2 to $85 \pm 2\ M^{-1}$ when H_2O was replaced by D_2O. This large isotope effect, $K_{sv}(H_2O)/K_{sv}(D_2O) \approx 2$, confirms the participation of the tyrosyl phenolic group in the quenching mechanism.

Tryptophan fluorescence can be quenched by tyrosine. Since a large number of proteins contain tryptophan residues, fluorescence quenching experiments provide an easy and convenient tool to investigate the molecular interactions between peptides containing tyrosine and proteins.

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REFERENCES

1. T. Peters, Jr. Serum albumin. In C. B. Enfinsen, J. T. Edsall, and F. M. Richards (eds.), *Advances in Protein Chemistry*, Vol. 37, Academic Press, New York, 1985, pp. 161–245.
2. B. Malfroy, J. P. Swerts, A. Guyon, B. P. Roques, and J. C. Schwartz. High-affinity enkephalin-degrading peptidase in brain is increased after morphine. *Nature* 276:523–526 (1978).
3. F. W. J. Teale. The ultraviolet fluorescence of proteins in neutral solution. *Biochem. J.* 76:381–388 (1960).
4. J. R. Lakowicz and H. Cherek. Dipolar relaxation in proteins on the nanosecond timescale observed by wavelength-resolved phase fluorometry of tryptophan fluorescence. *J. Biol. Chem.* 255(3):831–834 (1980).
5. N. J. Turro. *Modern Molecular Photochemistry*, Benjamin-Cummings, Menlo Park, CA, 1974.
6. J. P. Laussac and B. Sarker. Characterization of copper(II) and nickel(II)—Transport site of human serum albumin. Studies of copper(II) and nickel(II) binding to peptide 1-24 of human serum albumin by ^{13}C and ^{1}H NMR spectroscopy. *Biochemistry* 23:2832–2838 (1984).
7. J. W. Longworth. Intrinsic fluorescence of proteins. In R. B. Cundall and R. E. Dale (eds.), *Time Resolved Fluorescence Spectroscopy in Biochemistry and Biology*, NATO ASI Series, Series A: Life Sciences, Vol. 89, Plenum Press, New York, 1983, p. 651.