Proc. Indian Acad. Sci. (Chem. Sci.), Vol. 107, No. 1, February 1995, pp. 79–86.

Selective quenching of tryptophanyl fluorescence in bovine serum albumin by the iodide ion

MEENAKSHI MARUTHAMUTHU* and G SELVAKUMAR

Department of Physical Chemistry, University of Madras, Guindy Campus, Madras 600 025, India

MS received 10 February 1994; revised 10 January 1995

Abstract. Modified Stern–Volmer equation is obeyed by bovine serum albumin (BSA)–iodide system showing selective quenching of tryptophanyl fluorescence of BSA. The fraction of accessible protein fluorescence is 0·56 and the effective Stern–Volmer constant is 29·0 M^{-1} at *p*H 7·4 in 0·005 M phosphate buffer at 25°C. Collisional quenching is operative both in the BSA–I^- system and the model system, tryptophan-I^-. It is supported by the observed relationship between the ratio of quenching rate constants (k_q) and diffusion coefficients and also k_q with bulk viscosity.

Keywords. Tryptophanyl fluorescence; selective quenching; bovine serum albumin; iodide; modified Stern–Volmer equation.

1. Introduction

In the case of proteins, the fluorophoric aminoacid residues are phenylalanine, tyrosine and tryptophan. Of these fluorophores, the tryptophan fluorophore plays a vital role in protein fluorescence. In the study of the solution structure of multi-tryptophan proteins, selective fluorescence quenching is used to map out the exposed and buried tryptophan residues. Lehrer's work on lysozyme–iodide system (Lehrer 1971) showed iodide to be a selective quencher in probing fluorophore exposure and also initiated topographical studies based on fluorescence quenching involving various protein-quencher systems. Since the mechanism of fluorescence quenching should be collisional in order to probe the exposed and accessible fluorophores, such quenching probes are limited. The successful use of iodide as a selective quencher stems from its charge, heavy hydration and hence inability to penetrate the interior of the protein matrix besides collisional quenching.

Bovine serum albumin (BSA) is a very good example of a simple multi-tryptophan protein with only two tryptophan (Trp) residues. Trp 134 in loop 3 and Trp 212 in loop 4 of BSA are topographically distinct (Peters 1985) in that Trp 134 is apparently more exposed and Trp 212 is conserved or buried in the interior. In the case of such a heterogeneity of the Trp fluorophore in BSA, the quencher probe iodide is expected to quench selectively only the exposed Trp fluorophore in BSA and this is examined by investigating the BSA–iodide system. Tryptophan–iodide system is also examined as a model for the protein–iodide system.

*For correspondence

2. Experimental

Fluorescence intensity measurements have been made using Aminco-Bowman spectrophotofluorometer using the excitation wavelength of 295 nm and emission wavelength of 350 nm for the BSA–iodide system. The excitation wavelength is chosen so as ensure selective excitation of Trp residue in the protein. In the case of tryptophan–iodide model system, 284 nm and 360 nm are chosen as the wavelengths of excitation and emission respectively. The absorbance of the samples at the excitation wavelength is $< 0{\cdot}1$ and hence no correction for inner filter effects (Quay *et al* 1985) is necessary.

All the experiments have been performed at *p*H 7·4 in phosphate buffer of concentration 0·005 M and 25°C. Such a low concentration of phosphate buffer has been chosen in order to minimise the quenching effect by phosphate as phosphate is also a quencher of protein fluorescence (Cowgill 1963). Sodium chloride has been used for the maintenance of a constant ionic strength (μ) of 0·2 M. BSA (a fatty acid-free sample from Sigma Chemical Co., USA) has been used as such. The concentration of BSA employed in the quenching experiments ranges from 0·3 to 1·5 μM, [BSA] being determined spectrophotometrically using the molar extinction coefficient of $4{\cdot}4 \times 10^4\,M^{-1}cm^{-1}$ at 279 nm. KI is the source of iodide and $[I^-]$ ranges from 0·005 M to 0·2 M. $[Na_2S_2O_3] = 5 \times 10^{-4}$ M is always maintained in the experimental solution to prevent the formation of triiodide. This is essential since I_3^- absorbs in the wavelength region of tryptophanyl fluorescence. For the model system investigated, [tryptophan] ranges from 3 μM to 15 μM.

3. Results and discussion

3.1 *BSA–iodide system*

Heavy atom effect in conjunction with the discriminating effect of iodide between the exposed and buried fluorophores in proteins make iodide a suitable quencher probe. The fluorescence emission spectra of BSA at *p*H 7·4 in phosphate buffer in the absence and presence of iodide show the quenching effect of iodide. The decrease in fluorescence intensity with increasing iodide concentration in evident from the quenching profiles corresponding to various concentrations of iodide (0·05 to 0·2 M). No significant conformational changes in the protein induced by iodide are also evident as the fluorescence emission maximum of BSA is the same even in presence of iodide. The quenching data for BSA–iodide system were analysed using Stern–Volmer equation,

$$F_0/F = 1 + K_q[Q], \tag{1}$$

where F_0 is the fluorescence intensity in the absence of the quencher, F, the fluorescence intensity in presence of the molar concentration of the quencher $[Q]$ and K_q is the Stern–Volmer quenching constant. The Stern–Volmer plot of F_0/F vs $[I^-]$ is found to be nonlinear (figure 1) with negative deviation and this is characteristic of multi-fluorophore proteins with unequal accessibility of the fluorophores to quenching. A nonlinear Stern–Volmer plot with downward curvature in BSA–iodide system indicates the heterogeneity of fluorophores, i.e., two classes of Trp fluorophores with unequal accessibility to quenching by iodide. Since there are only

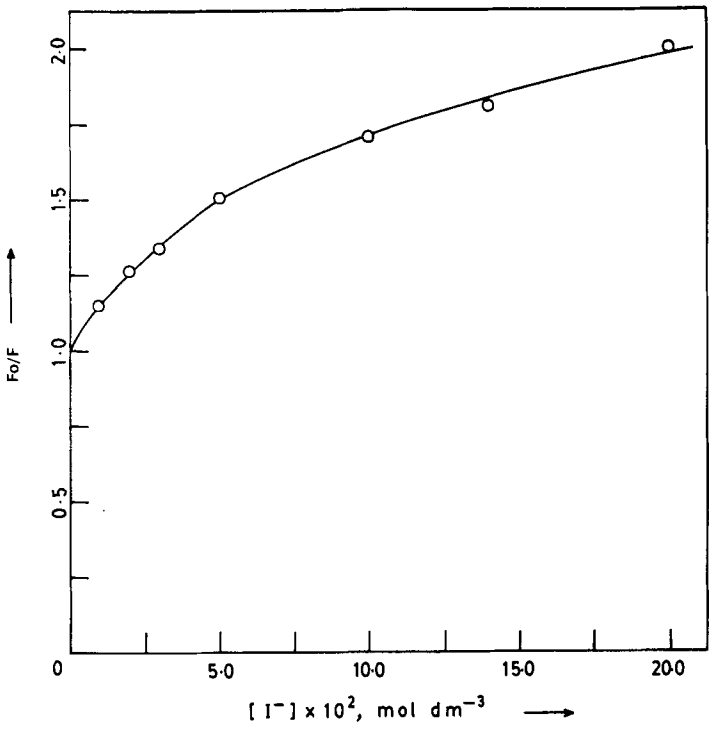

Figure 1. Stern–Volmer plot with negative deviation for the quenching of tryptophanyl fluorescence of BSA by iodide. [BSA] = 0·6 μM, pH = 7·4 [$Na_2S_2O_3$] = 5×10^{-4} M, μ = 0·2 M and 25°C. $\lambda_{excitation}$ = 295 nm, $\lambda_{emission}$ = 350 nm.

two Trp fluorophores in BSA, one Trp residue is accessible to quenching while the other is inaccessible. Modified Stern–Volmer equation (Lehrer 1971; Lehrer *et al* 1978) was therefore applied to such a heterogeneous system.

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a} + \frac{1}{f_a K_q [Q]}. \tag{2}$$

In the above equation, f_a stands for the fraction of accessible protein fluorescence and K_q, effective Stern–Volmer quenching constant, the other terms having the same significance as before. A plot of $F_0/(F_0 - F)$ vs [iodide]$^{-1}$ in accordance with the above equation is found to be linear with the intercept on the ordinate (figure 2). The reciprocal of the intercept gives the value of f_a while the intercept/slope gives the value of the effective quenching constant, K_q. Even though the modified Stern–Volmer plot corresponding to 0·6 μM BSA alone is presented, the fluorescence quenching data corresponding to three different concentrations of BSA afforded very nearly the same values of f_a and K_q when analysed by this method. The values of f_a and K_q as a function of [BSA] are presented in table 1 along with the mean values of the same. The mean value of f_a is found to be 0·56 indicating that nearly one-half of the protein fluorescence is accessible to quenching by iodide. This observation is in agreement with expectation. The accessible Trp residue must be the exposed Trp residue of BSA, namely Trp 134 in loop 3. The other Trp residue, Trp 212 in loop 4 is not accessible to

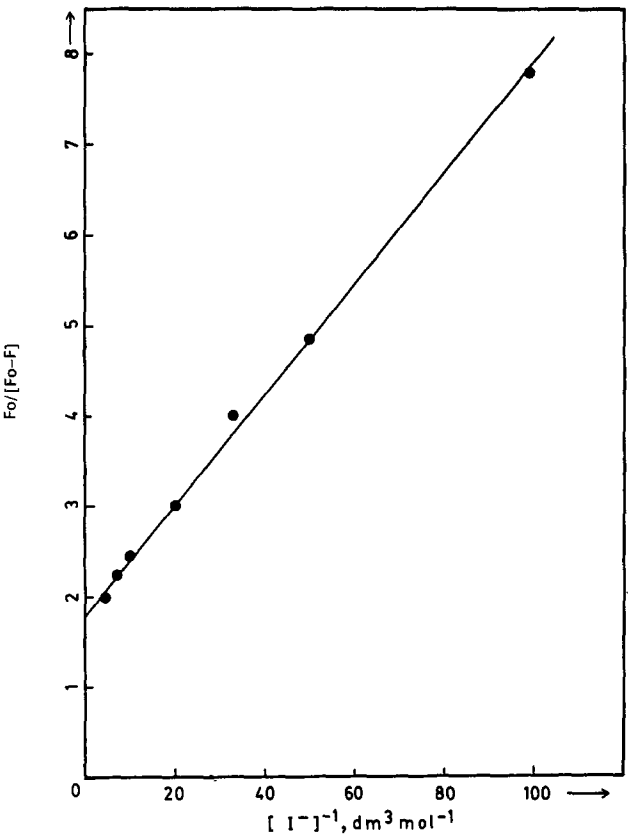

Figure 2. Modified Stern–Volmer plot for tryptophanyl fluorescence quenching in BSA by iodide. [BSA] = 0·6 μM, *p*H = 7·4 and 25°C.

Table 1. Quenching parameters for BSA–I^- system at *p*H 7·4 in phosphate buffer of 0·005 M, μ = 0·2 M and 25°C.

[BSA] (μM)	f_a	K_q (M^{-1})	$k_q(\times 10^9)$ ($M^{-1}s^{-1}$)
0·3	0·55	28·2	4·2
0·6	0·56	29·7	4·5
1·2	0·56	29·0	4·4
Mean value	0·56	29·0	4·4

quenching by iodide since the burial of this Trp residue somewhat in the interior of the protein matrix makes the collisional quencher ineffective as far as the sterically shielded Trp fluorophore is concerned. The mean value of the effective quenching constant, K_q, for BSA–iodide system is found to be 29·0 M^{-1} (table 1). Making use of the literature value (Luk 1971) of the fluorescence lifetime of BSA in the absence of the

quencher, $\tau_0 = 6{\cdot}65$ ns, the bimolecular quenching rate constant k_q for BSA– iodide system can be evaluated as $K_q = k_q\tau_0$. Thus, k_q of $4{\cdot}4 \times 10^9\,M^{-1}s^{-1}$ is obtained for iodide quenching of Trp fluorescence of BSA and this actually is the effective k_q for the accessible Trp residue in BSA. Alternatively, an approximate value of $(k_q)_{eff}$ can also be inferred making use of the following equation due to Lehrer (1971).

$$(k_q)_{eff} = \frac{(K_q)_{eff}\, n_a}{n_T \tau_0 f_a} \tag{3}$$

where n_a is the number of accessible Trp side-chains and n_T is the total number of Trp residue per macromolecule. In this way, $(k_q)_{eff} = 3{\cdot}9 \times 10^9\,M^{-1}s^{-1}$ is obtained for the system. The observed value of k_q is in good agreement with those observed for tryptophan and its derivatives with zero net charge investigated by Lehrer (1971) under similar experimental conditions.

The binding of iodide to BSA (Scatchard *et al* 1957) is quite facile with binding constants of the order of $9{\cdot}2 \times 10^3\,M^{-1}$. Hence one would expect bound iodide to participate in quenching and hence static quenching. However, there is no upward curvature or positive deviation in the Stern–Volmer plots for the BSA–I^- system showing the absence of any static quenching. It can be inferred therefore that tryptophan residue in BSA is not involved in binding with iodide.

The selective quenching of only the exposed Trp residue in BSA and the magnitude of the quenching rate constant also indicate the absence of any long-range energy transfer between the Trp residues.

3.2 *Tryptophan–iodide system*

Despite the detailed investigation of this model system by Lehrer (1971) for the lysozyme–iodide system, the use of phosphate buffer in our work in contrast to the organic buffer, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid buffer (or HEPES) employed in Lehrer's work has led to the study of the model system under experimental conditions similar to those employed in the BSA–iodide system. The experimental conditions correspond to *p*H 7·4 using 0·005 M phosphate buffer, $\mu = 0{\cdot}2$ M (using NaCl), $[Na_2S_2O_3] = 5 \times 10^{-4}$ M, 25°C, [Trp] = 3 to 15 μM and $[I^-]$ 0·05 to 0·4 M in the model system. Quenching profiles for Trp–I^- system at constant [Trp] and various concentrations of iodide show iodide quenching of Trp fluorescence of L-tryptophan. The Stern–Volmer plot for the Trp–I^- system is linear (figure 3), consistent with the Stern–Volmer equation for collisional quenching. The quenching constant, K_q, evaluated from the slope of the above plot is $14{\cdot}5\,M^{-1}$. This value is higher than that observed by Lehrer (1971), $11{\cdot}6\,M^{-1}$ for the system in HEPES buffer and also the value of $11{\cdot}7\,M^{-1}$ reported by Edwards and Salva (1986). However, a higher value of K_q, $18{\cdot}9\,M^{-1}$ for the Trp–I^- system in 0·025 M phosphate buffer of *p*H 7·0 has been obtained by Meyers and Seybold (1979). The higher magnitude of the Stern–Volmer constant in our work and in the above mentioned work can therefore be ascribed to the additional quenching effect of phosphate besides that of iodide as phosphate is also a quencher of Trp fluorescence (Cowgill 1963).

The fluorescence lifetime of L-tryptophan in the absence of the quencher, reported by Chen *et al* (1967), $\tau_0 = 2{\cdot}6$ ns is utilized to get the bimolecular quenching rate constant k_q for the Trp–I^- system. Thus we obtain $k_q = 5{\cdot}6 \times 10^9\,M^{-1}s^{-1}$ for the

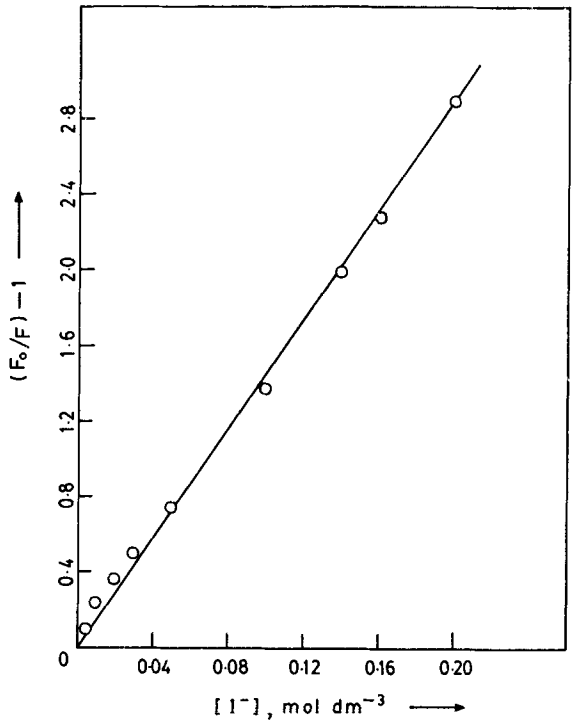

Figure 3. Stern–Volmer plot for the tryptophan–I^- system. [Trp] = 10 μM, pH = 7·4, [$Na_2S_2O_3$] = 5×10^{-4} M, μ = 0·2 M at 25°C, $\lambda_{excitation}$ = 284 nm and $\lambda_{emission}$ = 360 nm.

model system and the value closely parallels the ones observed for indole, tryptophan and their derivatives (Lehrer 1971). The diffusion-controlled rate constant for quenching can be calculated making use of the following equation,

$$k_{diff} = 4\pi RDN/1000 \tag{4}$$

wherein R, the interaction distance, is the sum of the molecular radii of the fluorophore and the quencher ($R \simeq 5$A), D is the sum of their diffusion coefficients and N, the Avogadro number. The diffusion coefficients of L-tryptophan and iodide are $0{\cdot}55 \times 10^{-5}$ cm^{2}s^{-1} and $2{\cdot}0 \times 10^{-5}$ cm^{2}s^{-1} respectively (Othmer and Thakar 1953; Harned and Owen 1958) and hence D is equal to $2{\cdot}55 \times 10^{-5}$ cm^{2}s^{-1}. Considering the interaction distance to be about 5Å, k_{diff} is found to be $9{\cdot}65 \times 10^9$ M^{-1}s^{-1} on the basis of the above relation. The observed value of k_q, $5{\cdot}6 \times 10^9$ M^{-1}s^{-1} is less than k_{diff} showing that the collisional encounters of the ionic quencher, iodide, with the Trp fluorophore are reduced due to the presence of the anionic carboxylate group in tryptophan at the experimental pH of 7·4. Even though the amino group in Trp would exist in the cationic form as $\overset{+}{N}H_3$ only, at this pH, it is probably the repelling effect of carboxylate that dominates and thus the reduced quenching efficiency (γ) of iodide γ, defined as

$$\gamma = k_q/k_{diff} \tag{5}$$

according to Eftink and Ghiron (1981), is found to be 0·58 for Trp–I^- system showing that the probability of deactivation of the excited singlet state of Trp during the collisional encounter with I^- is 58% only.

Collisional or dynamic quenching can also be elucidated with the linear dependence of k_q on T/η where T is the absolute temperature and η is the bulk viscosity. Since a constant temperature of 298 K has been maintained in the quenching experiments, the inverse dependence of k_q on η is illustrated from the constancy of the product of k_q and η or equivalently the product of K_q/F_0 and η. Stern–Volmer constant for Trp–I^- system at *p*H 7·4 in 15% glycerol is found to be 12·0 M^{-1} and the following data showing the constancy of $(K_q/F_0) \times \eta$, within the limits of experimental error, (table 2) for water and 15% glycerol also support collisional quenching.

3.3 *Comparison of BSA–I^- system with the model system*

Selective quenching of the exposed Trp in BSA by iodide is inferred with the use of the modified Stern–Volmer equation, the quenching parameters being $f_a = 0{\cdot}56$, $K_q = 29{\cdot}0\ M^{-1}$ and $k_q = 4{\cdot}4 \times 10^9\ M^{-1}s^{-1}$. Comparing the k_q values of BSA–I^- and Trp–I^- systems, the lower value of k_q in the case of the macromolecular system can be ascribed to the smaller diffusion coefficient of the protein since D_{BSA}, diffusion coefficient of BSA would be at least an order of magnitude smaller (Tanford 1961) than that for the low molecular weight compounds. The ratio of k_q values for the two systems should be equal to the ratio of diffusion coefficients as given below,

$$\frac{k_q \text{ for Trp–}I^- \text{ system}}{k_q \text{ for BSA–}I^- \text{ system}} = \frac{D_{Trp} + D_{I^-}}{D_{BSA} + D_{I^-}} \tag{6}$$

Thus, the ratio of k_q values is found to be 1·27. The smaller values of D_{BSA} relative to D_{I^-} leads to the relationship, $D_{BSA} + D_{I^-} \simeq D_{I^-}$ and hence the ratio of the diffusion coefficients, $(D_{Trp} + D_{I^-})/D_{I^-}$ turns out to be $2{\cdot}55 \times 10^{-5}/2{\cdot}0 \times 10^{-5} = 1{\cdot}275$, in good agreement with the ratio of k_q values. The above correlation of k_q values with the diffusion coefficients also supports the collisional quenching mechanism operative both in Trp–I^- and BSA–I^- systems.

Stern–Volmer constant for BSA–I^- system is very much greater relative to that for the model system. In fact K_q for BSA–I^- system is twice that for Trp–I^- system. The presence of positively charged aminoacid residues like arginine and lysine in BSA explains the higher value of K_q in the macromolecular system. High values of Stern–Volmer constants as well as quenching rate constants have been observed for Trp fluorescence quenching in random sequence polypeptides containing lysine residues by iodide by Lehrer (1971).

Table 2. Constancy of $(K_q/F_0) \times \eta$ in water and 15% glycerol to illustrate collisional quenching in Trp–I^- system.

Solvent	$\eta \times 10^2$ (poise)	K_q (M^{-1})	K_q/F_0 (M^{-1})	$(K_q/F_0) \times \eta$ (M^{-1} poise)
Water	1·00	14·5	26·0	0·26
15% Glycerol	1·45	12·0	18·6	0·27

Despite the binding of I^- to BSA (Scatchard *et al* 1957), static quenching is not observed in BSA–I^- system showing that Trp is not part of the binding site in the protein. Trp–I^- system exhibits neither binding nor static quenching.

f_a should be equal to 0·5 to account for the selective quenching of Trp residues in BSA. However the observed value of f_a is $0{\cdot}56 \pm 0{\cdot}01$, slightly greater than 0·5. The difference can be accounted for by experimental errors. It can also be explained by considering the dynamic exposure of the buried Trp residue due to conformational fluctuations (Weber 1975) in the protein in aqueous solution. The periodic exposure of the buried Trp residue in BSA may account for f_a being slightly higher than 0·5.

References

Chen R F, Vurek G G and Alexander N 1967 *Science* **156** 949
Cowgill R W 1963a *Arch. Biochem. Biophys.* **100** 36
Cowgill R W 1963b *Biochem. Biophys. Acta* **75** 272
Edwards A M and Salva E 1986 *Radiat. Environ. Biophys.* **25** 113
Eftink M R and Ghiron C A 1981 *Anal. Biochem.* **114** 199
Harned H S and Owen B B 1958 *The physical chemistry of electrolytic solutions* (New York: Reinhold)
Lehrer S S 1971 *Biochemistry* **10** 3254
Lehrer S S and Leavis P C 1978 in *Methods in enzymology* (eds) C H W Hirs and S N Timasheff (New York: Academic Press) vol. 49, p. 222
Luk C K 1971 *Biopolymers* **10** 129
Meyers M L and Seybold P G 1979 *Anal. Chem.* **51** 1609
Othmeer D F and Thakar M S 1953 *Ind. Eng. Chem.* **45** 589
Peters T Jr 1985 in *Advances in protein chemistry* (eds) C B Anfinsen, J T Edsall and F M Richards (New York: Academic Press) vol. 37, and references cited therein
Quay S C, Heropoulos A, Commes K and Dzau V J 1985 *J. Biol. Chem.* **260** 15055
Scatchard G, Coleman J and Shen A 1957 *J. Am. Chem. Soc.* **79** 12
Tanford C 1961 *Physical chemistry of macromolecules* (New York: Wiley)
Weber G 1975 *Adv. Protein Chem.* **29** 1