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FLUOROMETRIC CHARACTERIZATION OF DITYROSINE: COMPLEX FORMATION WITH BORIC ACID AND BORATE ION [1]

Dean A. Malencik and Sonia R. Anderson*

Department of Biochemistry and Biophysics
Oregon State University
Corvallis, Oregon 97331-6503

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Borate/boric acid solutions have distinctive effects on the absorption and fluorescence emission spectra of dityrosine. In the presence of excess borate/boric acid, the fluorescence emission maximum of the singly ionized dityrosine chromophore shifts from 407 nm (quantum yield = 0.80) to 374 nm (quantum yield = 0.14). Fluorescence measurements performed as a function of pH and concentration are consistent with a 1:1 complex which may dissociate to either boric acid and singly ionized dityrosine (K_1 = 17 mM) or to monoborate ion and unionized dityrosine (K_2 = 0.10 mM). As a consequence of the pK_a values characteristic of dityrosine and boric acid, complex formation is maximal near pH 8. 2,2'-Dihydroxy-biphenyl shows similar interactions. The fluorescence of dityrosyl calmodulin (0 Ca^{2+}) also responds to the addition of boric acid, giving K_1 = 42 mM and K_2 = 2 mM. Singly ionized dityrosine produced through dissociation occurring in the excited state does not interact with boric acid.

Dityrosine is an amino acid derivative that exhibits intense fluorescence with an emission maximum near 400 nm. Phenolic coupling leading to protein dityrosine is a normal post-translational process in some cases and a consequence of environmental stress in others (1). Dityrosine formation may result from exposure to oxygen radicals (2), γ-irradiation (3), or ultraviolet irradiation (4).

Several years ago we discovered that bovine brain calmodulin undergoes a calcium-dependent, photoactivated reaction leading to the cross-linking of its only two tyrosine residues--Tyr^{99} and Tyr^{138}. Steady state fluorescence intensity and anisotropy

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*Address correspondence to this author.

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measurements performed at 400 nm revealed diminished interactions of the cross-linked calmodulin molecule with both calcium and enzymes (5). During chemical characterization of UV-irradiated calmodulin, we developed a simple high performance liquid chromatography procedure capable of giving a complete amino acid analysis that includes dityrosine and the three phospho-amino acids (6).

This report contains spectrophotometric and fluorometric measurements demonstrating the ability of dityrosine to associate reversibly with both boric acid and the monoborate ion. These interactions may find applications in the identification of dityrosine in protein hydrolysates and the probing of dityrosine cross-links in proteins.

MATERIALS AND METHODS

Corrected fluorescence emission spectra and intensity measurements were obtained with a Perkin Elmer LS-50 fluorescence spectrophotometer connected to a circulating constant temperature (25°C) bath. Quantum yields were obtained by comparison of the integrated spectra to that of quinine sulfate, which has a quantum yield of 0.7 (7). The absorbancies of the dityrosine samples and of quinine were matched (A = 0.05) at the excitation wavelength. Slit widths were usually fixed at 2.5 nm. Absorption spectra were recorded with the Perkin Elmer Lambda 3 absorption spectrophotometer. Quartz fluorescence cuvettes (1 cm path) were used routinely. Dityrosine (1) and cross-linked calmodulin (5) were prepared and purified according to published procedures. 2,2'-Dihydroxy-biphenyl was purchased from Fluka Chemicals. All solutions were made up from reagent grade or best available grade chemicals and distilled water that had been purified in a Millipore reagent water system. pH measurements were performed with a Corning model 125 pH meter. The addition of small volumes of KOH or HCl (total dilution <5%) maintained individual pH values within ±0.01 (Table 1).

RESULTS

Dityrosine has a characteristic pH-dependent absorption spectrum (4) which we routinely employ to check concentrations. We thus discovered that the spectra of dityrosine obtained with alkaline borate buffers differ from those obtained with alkaline carbonate, phosphate, or NaOH solutions (Figure 1A). The fluorescence spectra show a change in emission maximum from 407 nm to 374 nm and a decrease in quantum yield from 0.80 to 0.14 when excess boric acid/borate is present (Figure 1B).

Note that wavelengths can be selected ($\lambda_{ex} \geq 325$ nm; $\lambda_{em} \geq 420$ nm) that allow monitoring of the 407 nm-emitting species with negligible background due to the proposed

Table 1. Properties of Dityrosine, 2,2'-Dihydroxy-biphenyl, and Dityrosyl Calmodulin

	dityrosine	2,2'-dihydroxy -biphenyl	dityrosyl calmodulin
emission maximum (nm) (no additions, $\lambda_{ex} \geq 320$ nm)	407	396	402[1]
quantum yield (no additions, $\lambda_{ex} \geq 320$ nm)	0.80	0.97	--
phenolic pK_a (λ_{ex} = 315-325 nm)	7.0	7.3	7.88 (EGTA)[1] 7.59 (3 mM Ca^{2+})
emission maximum (nm) (0.5 M borate/boric acid, λ_{ex} = 285 nm)	374	--	--
quantum yield (0.5 M borate/boric acid, λ_{ex} = 285 nm)	0.14	--	--
K_1 (mM H_3BO_3)	16.6±0.2	15.9	42 (EGTA)
K_2 (mM $B(OH)_4^-$)	0.11±0.01	0.20	2.0 (EGTA)

Solutions contained 0.10 M KCl. Temperature: 25°C.

[1]From Malencik & Anderson (5).

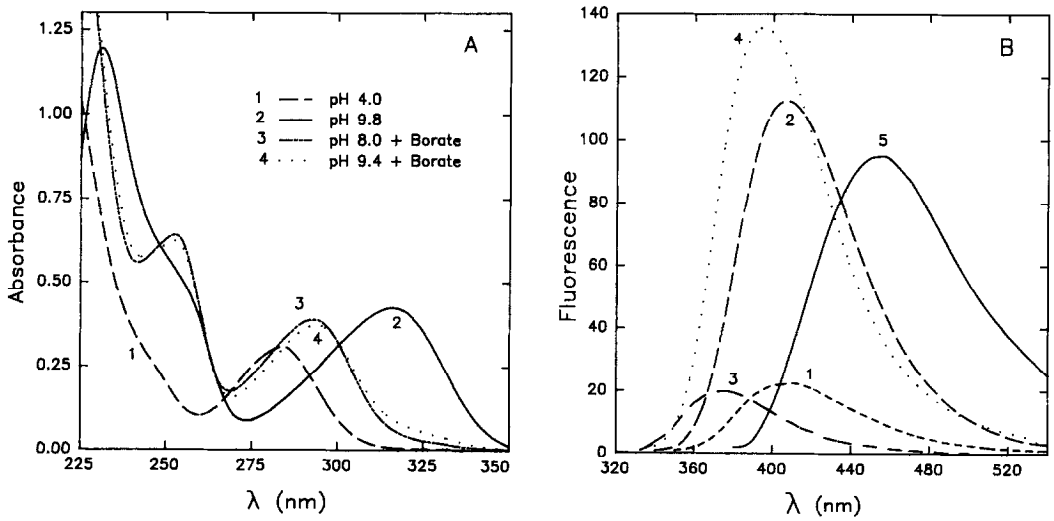


Figure 1. A. Ultraviolet absorption spectra of aqueous 73 μM solutions of dityrosine containing 5 mM acetic acid, 0.1 M KCl, pH 4.0 (#1), 5 mM sodium phosphate, 0.1 M KCl, pH 9.8 (#2), 0.5 M potassium borate, 0.1 M KCl, pH 8.0 (#3), and 0.5 M potassium borate, 0.1 M KCl, pH 9.4 (#4). B. Fluorescence emission spectra of dityrosine in 5 mM acetic acid, 0.1 M KCl, pH 4.5 with λ_{ex} = 285 nm (#1); in 5 mM Tris, 0.1 M KCl, pH 8.7 with λ_{ex} = 315 nm (#2); and in 0.5 M potassium borate, pH 8.7 with λ_{ex} = 285 nm (#3). Spectrum #4 was obtained with 2,2'-dihydroxy-biphenyl in 5 mM Tris, 0.1 M KCl, pH 8.7 with λ_{ex} = 315 nm. Spectrum #5 represents the normalized emission spectrum of quinine in 0.10 M H_2SO_4 with λ_{ex} = 285 nm or 315 nm. The absorbance at the excitation wavelength was fixed at 0.05.

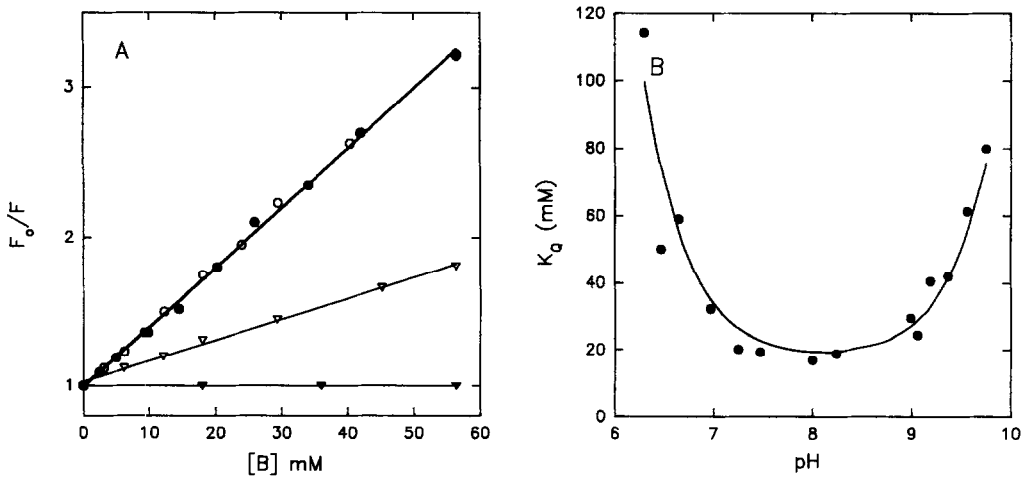


Figure 2. A. Examples of the quenching of dityrosine fluorescence by borate/boric acid solutions. [B] is the sum of the concentrations of H_3BO_3 and $B(OH)_4^-$. F_o is the fluorescence intensity determined at [B] = 0. Dityrosine (5 μM) was examined in 2 mM sodium carbonate, 0.1 M KCl, pH 9.06, λ_{ex} = 325 nm (○) or in 0.10 M acetic acid - KOH, pH 4.0, λ_{ex} = 285 nm (▾). All intensities were determined at an emission wavelength of 425 nm. Also shown are results obtained with dityrosyl calmodulin (▿) in 2 mM potassium phosphate, 1 mM EGTA, 0.10 M KCl, pH 8.52 and 2,2'-dihydroxy-biphenyl (●) in 2 mM potassium phosphate, 0.10 M KCl, pH 7.57, λ_{ex} = 325 nm. The straight lines were calculated for K_Q = 24.4 mM (○), K_Q = ∞ (▾), K_Q = 62 mM (▿), and K_Q = 25 mM (●). B. Dependence of K_Q on pH for dityrosine. The smooth curve was calculated for the equilibrium constants summarized in Table 1 and pK_a = 9.2 for boric acid.

borate complex. Representative effects of varying borate/boric acid concentration on the fluorescence intensities of dityrosine, 2,2'-dihydroxy-biphenyl, and dityrosyl calmodulin (+EGTA) are shown in Figure 2A. The linear relationship between F_o/F (where F_o is the intensity obtained in the absence of the ligand and F is that obtained in its presence) and [B] (sum of the concentrations of boric acid and borate ion) is described by the equation:

$$F_o/F = 1 + [B]/K_Q. \qquad (1)$$

This relationship compares to the Stern-Volmer equation, which is applied to cases of collisional quenching. K_Q corresponds to the value of [B] at which a 50% decrease in fluorescence occurs. In the present case, involving a ground state complex detected in the absorption spectrum, the value of K_Q is determined entirely by true equilibrium constants.

The dependence of the values of K_Q on pH (Figure 2B) shows that the ionizations of boric acid and dityrosine need to be considered in quantative evaluation of complex formation. Since our choice of excitation and emission wavelengths excludes fluorescence

due to either excited state ionization or the borate complex, the observed intensity is directly proportional to the concentration of ground state singly ionized dityrosine. The total concentration of dityrosine ($[diY]_{Total}$) is the sum of the concentrations of the ionized species ($[diY^-]$), unionized dityrosine ([diY]), and the complex ([BdiY]). Thus

$$\frac{F_o}{F} = \frac{[diY]_{Total} - [diY]_o}{[diY]_{Total} - [diY]_B - [BdiY]} . \qquad (2)$$

Subscripts O and B designate the concentrations of the unionized species in the absence and presence of borate/boric acid, respectively. Since the borate/boric acid concentrations are in excess of the concentration of dityrosine (generally fixed at 2 μM to 10 μM), [B] = $[H_3BO_3]$ + $[B(OH)_4^-]$.

Assuming that no other reactions contribute to the value of K_Q, the equilibria in question are:

a. The ionization of boric acid.

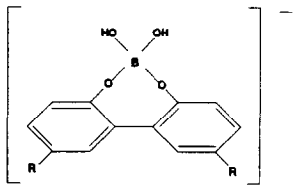
$H_3BO_3 + H_2O \leftrightarrows B(OH)_4^- + H^+ \quad K_B = 6.3x10^{-10}$ M

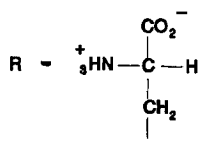
b. The phenolic ionization of dityrosine.

Dityrosine $\leftrightarrows$ Dityrosine$^-$ + H^+ $\quad K_{diY} = 1x10^{-7}$ M

Experiments with 2,2'-dihydroxy-biphenyl had indicated that there is no further ionization up to pH 13 (8).

c. The dissociation of the complex, which has the following schematic structure predicted from the association of borate with organic polyhydroxy compounds (9).





$$\text{Complex}^- + H_2O \underset{K_1}{\leftrightarrows} H_3BO_3 + \text{Dityrosine}^-$$

$$\text{Complex}^- + 2H_2O \underset{K_2}{\leftrightarrows} B(OH)_4^- + \text{Dityrosine}$$

$$\text{Complex}^- + H_2O + H^+ \underset{K_3}{\leftrightarrows} H_3BO_3 + \text{Dityrosine}$$

$$\text{Complex}^- + H_2O + OH^- \underset{K_4}{\leftrightarrows} B(OH)_4^- + \text{Dityrosine}^-$$

Note that only three of the six dissociation constants are independent.

$$K_1/K_2 = K_{diY}/K_B \qquad K_3 = K_1/K_{diY} \qquad K_4 = K_1K_B \times 10^{14}$$

Substitution of the concentrations terms in equation 2 gives

$$\frac{F_o}{F} = 1 + \frac{K_{diY}[H^+]\,[B]}{K_1\,([H^+]+K_{diY})\,([H^+]+K_B)}. \qquad (3)$$

In other words,

$$K_Q = \frac{([H^+]+K_{DiY})\,([H^+]+K_B)\,K_1}{K_{DiY}[H^+]}. \qquad (4)$$

Since calculation of K_1 requires values of K_{diY}, we re-determined the fluorescence intensity of dityrosine as a function of pH--employing λ_{ex} = 320 nm and λ_{em} = 400 nm (Figure 3). The results are consistent with pK_a = 7.0 for ground state dityrosine and pK_a = 7.3 for 2,2'-dihydroxy-biphenyl. We had previously reported that the pK_a of dityrosine is 7.1 (5).The smooth curve in Figure 2B represents a fit of the K_Q values obtained over the pH range of 6.3 to 9.8 with $K_{diY} = 10^{-7}$ M, $K_B = 6.3\times10^{-10}$ M, and K_1 = 16.6 mM boric acid. The deduced value of K_2 is 0.105 mM borate. Excluding the results obtained at pH 6.5, the average value of K_1 is 16.6±2.0 mM. The calculated values of K_3 (1.7×10^2 M) and K_4 (1.0M) show that equilibria 3 and 4 are unfavorable. The single experiment performed with 2,2'-dihydroxy-biphenyl corresponds to K_1 = 15.9 mM boric acid and K_2 = 0.20 mM borate.

Although $K_2 << K_1$, the association of singly ionized dityrosine with boric acid prevails experimentally as a result of the characteristic pK_a values. Dityrosine also ionizes in the excited state (4). pH titrations monitored at an exciting wavelength (285 nm) near the absorbance maximum of unionized dityrosine (Figure 3) suggest an excited state pK_a less than 2. The addition of varying concentrations of boric acid (up to 230 mM) to solutions of dityrosine from which all of the observed fluorescence is due to excited state ionization (pH

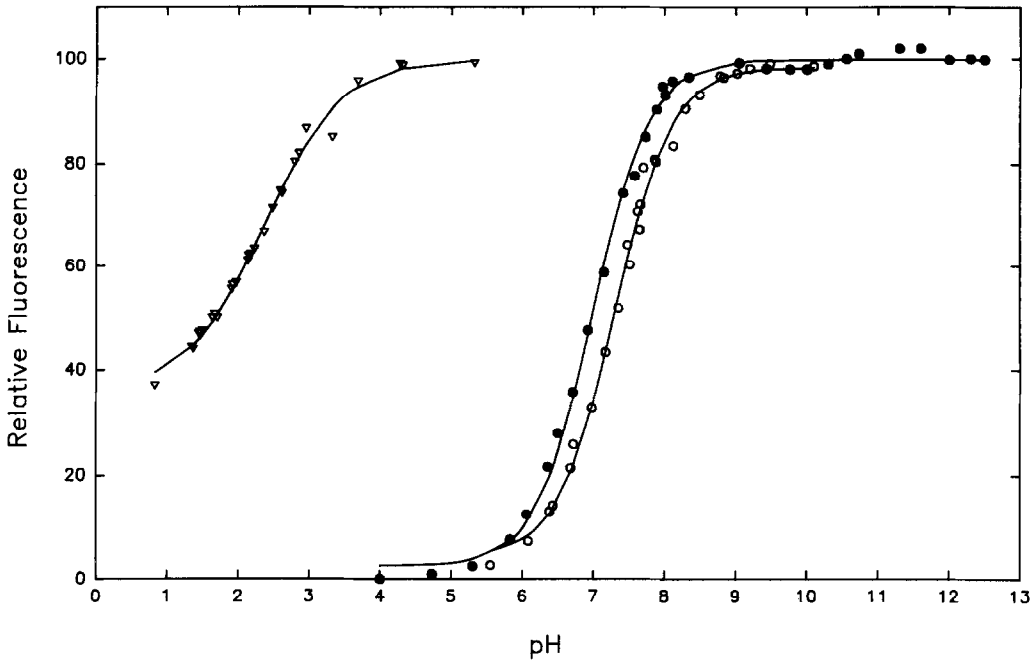


Figure 3. Ground state ionization of dityrosine (●) and of 2,2'-dihydroxy-biphenyl (○) was monitored in fluorescence measurements performed with λ_{ex} = 320 nm and λ_{em} = 400 nm. Conditions: 0.10 KCl, 5 mM Tris. Excited state ionization of dityrosine (▽) was determined with λ_{ex} = 285 nm, λ_{em} = 400 nm. Conditions: 50 mM H_2SO_4, 2 mM H_3PO_4.

4, λ_{ex} = 285 nm) results in no detectable interaction (Figure 2A). Apparently the excited state lifetime of dityrosine--4.3 ns (10)--is too small for appreciable complex formation to occur.

Dityrosyl calmodulin exhibits a weaker interaction with borate/boric acid than does free dityrosine. Considering that the pK_a of dityrosyl calmodulin is 7.88 in the absence of calcium (5), the values of K_1 and K_2 calculated from K_Q = 62 mM (pH 8.52) are 42 mM and 2.0 mM, respectively. The precipitation of calcium borate prevented examination of the calcium-calmodulin complex.

DISCUSSION

The influence of pH and concentration on the interaction of dityrosine with borate/boric acid solutions is consistent with a 1:1 diester complex which may dissociate to either boric acid and singly ionized dityrosine (K_1 = 17 mM) or to the monoborate ion and unionized dityrosine (K_2 = 0.1 mM). Fluorescence measurements reveal maximum interaction near pH

8 (Figure 2B), where about 90% of the dityrosine is in the ionized form and 90% of the boric acid is unionized. Thus, as a consequence of the characteristic pK_a values, the association of boric acid with singly ionized dityrosine prevails experimentally--even though $K_2 << K_1$.

The values of the dissociation constants can be compared to those reported for other complexes of diols with borate ion. For example--with 1,2-ethanediol, K_d = 0.46 M; with cis-1,2-cyclopentanediol, K_d = 38.5 mM; with D-mannitol, K_d = 0.91 mM; and with catechol, K_d = 0.2 mM (9). Since most of these diols do not ionize, the K_d values correspond to our values of K_2. Thus the associations of 2,2'-dihydroxy-biphenyl, dityrosine, and catechol with borate give complexes of comparable stability--even though the hydroxyl groups are located on two different rings in the present case. Borate is also known to form 2:1 complex anions, containing two molecules of polyol and a borate ion. Considering the concentrations of dityrosine employed and the equilibrium constants of the known 2:1 complexes, this is unlikely to occur under our conditions.

The diminished interaction of dityrosyl calmodulin with borate/boric acid (K_1 = 42 mM, K_2 = 2 mM) may reflect on the accessibility of the dityrosyl cross-link to the solvent. It could also reflect limitations on the rotations about the biphenyl bond.

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