Emission Polarization Study of the Interaction of Stellacyanin with Tris(2,2′-bipyridine)osmium(II)

ANN M. ENGLISH* and CLAIRE M. ACHKOUTI

Enzymology Research Group, Departments of Chemistry and Biology, Concordia University, 1455 de Maisonneuve Blvd. West, Montreal, Que., H3G 1M8, Canada

Received September 24, 1985

Abstract

The association of $Os(bpy)_3^{2+}$ and stellacyanin was investigated by measuring the emission polarization of the excited state of the complex [$^*Os(bpy)_3^{2+}$]. At high protein concentration ($\geq$1 mM) $Os(bpy)_3^{2+}$ appears to bind weakly to reduced stellacyanin and to oxidized stellacyanin to a lesser extent. These results are consistent with those obtained in a previous kinetic study on the redox quenching of $^*Ru(bpy)_3^{2+}$ by stellacyanin [1]. On excitation at 480 nm, the limiting polarization (P_0) of the $^*Os(bpy)_3^{2+}$ emission at 715 nm is 0.100. From P_0 we concluded that (1) protein-bound $Os(bpy)_3^{2+}$ has considerable rotational freedom independent of the protein-probe complex, and (2) the emission dipole of $^*Os(bpy)_3^{2+}$ is at ~45° to the C_3 molecular axis.

Introduction

In an effort to elucidate the mechanisms of electron transfer in biological systems, reactions involving redox proteins and small inorganic complexes have been investigated extensively [1–3]. In many instances rate saturation was observed at high reagent concentration and this was most clearly demonstrated in the quenching of the electronically excited state of $Ru(bpy)_3^{2+}$ by the blue copper proteins stellacyanin, plastocyanin and azurin [1]. Plots of reciprocal lifetime *vs.* protein concentration level-off at high protein concentration for the Cu(I) proteins but remain linear for quenching by the Cu(II) proteins. Since the reductive quenching studies clearly indicate rate saturation, we decided to directly probe the existence of a protein–small molecule complex.

The binding of small luminescent molecules to proteins can be evaluated by monitoring emission polarization. This technique can be used to obtain binding constants [4] as well as the rotational relaxation time of the emitting species [5]. To date only fluorescent organic molecules have been used in studies of this nature. Polarization measurements offer major advantages over equilibrium dialysis since only a small amount of protein is required and long equilibration times are not necessary.

The system we chose to study was the binding of $Os(bpy)_3^{2+}$ to stellacyanin. Stellacyanin has the largest molecular weight (20 000) [6] of the blue copper proteins used in the quenching experiments; hence, binding of a luminescent probe to stellacyanin would give rise to the greatest emission polarization. Also, NMR studies [7, 8] have shown that plastocyanin has a binding site for $Cr(phen)_3^{3+}$ but similar studies have not been undertaken for either stellacyanin or azurin. $Os(bpy)_3^{2+}$ was used since it is structurally similar to $Ru(bpy)_3^{2+}$ and has a suitable excited state lifetime [9].

Experimental

$Os(bpy)_3^{2+}$ was prepared according to the published procedure and recrystallized twice from 50% ethanol:H_2O solutions [9]. *Rhus vernicifera* stellacyanin was isolated from an acetone extract by the method of Reinhammer (A_{280}/A_{604} = 5.8) [10]. All emission measurements were carried out in sodium phosphate buffer, pH 7.0, μ = 0.1 M, 20 °C. Stock solutions of stellacyanin and $Os(bpy)_3^{2+}$ were filtered with micropore filters (Nucleopore) to remove any fine particles. The concentration of the stock solutions was determined spectrophotometrically (stellacyanin, ϵ_{604} = 4080 M^{-1} cm^{-1}; $Os(bpy)_3^{2+}$, ϵ_{480} = 12 000 M^{-1} cm^{-1}) [9, 10], and suitable aliquots of these solutions were added to 3 × 3 mm fluorescence cuvettes. The Cu(I) samples were prepared by adding a few grains of sodium ascorbate to the cuvette. Emission polarization was measured using type HNP'B polarizers (Polaroid Corporation) on a Perkin-Elmer MPF-44B spectrofluorimeter interfaced to a Differential Corrected

*Author to whom correspondence should be addressed.

0020-1693/86/$3.50

Spectra Unit (DCSU-2). For each orientation of the polarizers, the DCSU averages 300 data points in 95 s and automatically records the emission polarization which permitted the polarization to be determined to the third decimal. The performance of the polarizers was checked by measuring the fluorescence polarization of 8-anilino-1-naphtalene sulfonate bound to apomyoglobin. The results obtained were in good agreement with the literature values [11].

The viscosity of the Cu(II) stallacyanin solutions was measured previously at room temperature using an Ostwald viscometer [12].

Theory [5]

The emission polarization P is given by:

$$(I_{\parallel} - I_{\perp}G)/(I_{\parallel} + I_{\perp}G) \quad (1)$$

where $I_{\perp}$ and $I_{\parallel}$ are the emission intensities polarized perpendicular and parallel to the vertical laboratory axis. The excitation light is polarized parallel to the vertical laboratory axis. The G factor corrects for the polarization response of the detector system which is dependent on the emission wavelength and slit width. This factor is $I_{\parallel}/I_{\perp}$ under linearly-polarized excitation perpendicular to the vertical laboratory axis.

The quantitative relationship between polarization, excited state lifetime (τ), and the average rotational relaxation time (ρ) for a spherical particle is given by the Perrin equation:

$$(1/P) - (1/3) = \{(1/P_0) - (1/3)\}\left(1 + \frac{3\tau}{\rho}\right) \quad (2)$$

P_0 is the limiting polarization in the absence of rotation, and ρ is defined by:

$$\rho = 3\eta V/RT \quad (3)$$

where η is the viscosity, V the molar volume, R the gas constant and T the absolute temperature. Hence, the emission polarization of the probe will vary with its excited state lifetime and its rotational relaxation time which is sensitive to the temperature and viscosity of the medium.

Polarization will also vary with the extent of binding of the probe to a macromolecule. This permits the binding constant to be determined from the dependence of the emission polarization on the concentration of the macromolecule. Following the treatment given in ref. 4, the binding constant (K) for the association, $St + Os(bpy)_3^{2+} \rightleftharpoons St{:}Os(bpy)_3^{2+}$, can be obtained from a modified Scatchard plot:

$$\bar{s}/[St] = K(1 - \bar{s}) \quad (4)$$

Here $\bar{s}$ is the fractional saturation of the ligand, *i.e.*, the number of moles of ligand bound per mole of ligand, and it is obtained from:

$$\bar{s} = (P - P_{min})/(P_{max} - P_{min}) \quad (5)$$

where P is the observed polarization, P_{min} and P_{max} are the polarization values for the free and bound probe, respectively, and $[St]$ is the concentration of the unbound protein. Thus, in order to use emission polarization as a measure of binding it is necessary to obtain a value for the polarization when all the complex is bound to the protein, P_{max}. This can be estimated from the Perrin equation if we know ρ for the protein–probe complex (which is assumed equal to ρ for the protein alone), and P_0 and τ for the probe. Since ρ for stellacyanin has not been measured experimentally, it was calculated using eqn. (3) assuming $V = V_h$, the hydrated volume of the protein [14]:

$$V_h = M(V_1 + \delta V_2) \quad (6)$$

Here M is the molecular weight (20000), V_1 is the partial specific volume of stellacyanin, δ is the hydration (0.35 g H_2O/g protein), and V_2 is the partial specific volume of water.

Results

Determination of P_0 for $Os(bpy)_3^{2+}$

The polarization of $Os(bpy)_3^{2+}$ emission in 100% glycerol at different temperatures is shown in Table I, and $1/P$ *vs.* T/η is plotted in Fig. 1. The correlation coefficient is ~0.996 indicating the validity of eqn. (2) for this system, and the limiting polarization, P_0, was determined to be 0.099. To ensure that concentration depolarization was not occurring under the conditions of Fig. 1, P_0 was determined for $Os(bpy)_3^{2+}$ concentrations within the range 10^{-4}–

TABLE 1. Polarization (P) of $Os(bpy)_3^{2+}$ (1.1×10^{-4} M) in 100% Glycerol at Different Temperatures[a, b]

Temperature (°C)	Viscosity[c] (cp)	$P(\sigma)$[d]
10	3900	0.090(5)
15	2330	0.079(5)
20	1490	0.071(6)
30	629	0.055(8)
40	284	0.033(5)

[a]The excitation and emission wavelengths were 480 and 715 nm, respectively; a 700 nm cut-off filter was placed in the emission path to prevent scattered light from reaching the detector. Both the emission and excitation slits were 20 nm, the amplifier gain was 3.0 and the response time 5 s. Under these conditions scattering was not observed. [b]The excited state lifetime of $Os(bpy)_3^{2+}$ is 46 ns in air-saturated glycerol from the ratio of its emission intensities in water and glycerol ($\tau(H_2O)$ = 19 ns) [9]. [c]Viscosities from ref. 13. [d]Figures in brackets are standard deviations in units of the last digit.

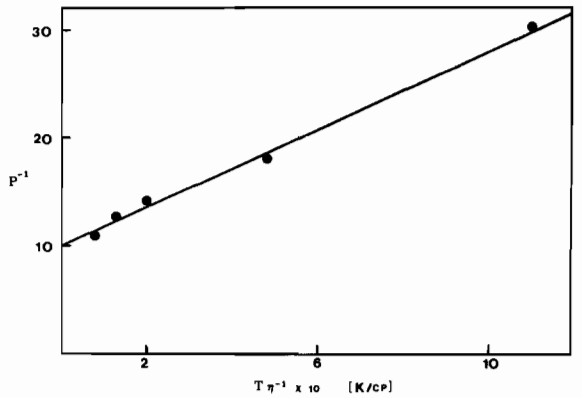

Fig. 1. Perrin plot ($1/P$ *vs.* T/η) for $Os(bpy)_3^{2+}$ (1.1×10^{-4} M) emission at 715 nm in 100% glycerol. From the y-intercept a value of 0.099 was obtained for P_0.

TABLE II. Emission Polarization of $Os(bpy)_3^{2+}$ in the Presence of Cu(II) and Cu(I) Stellacyanin[a]

[*St*] (mM)	Viscosity[b] (cp)	Cu(II)		Cu(I)	
		P_{obs}[c]	P_{corr}[d]	P_{obs}[c]	P_{corr}[d]
0	1.0	0.003(0.3)	0.003	0.001(1)	0.001
1.00	1.1	0.005(0.3)	0.005	0.007(4)	0.007
2.50	1.3	0.008(0.4)	0.007	0.012(3)	0.010
3.75	1.6	0.011(0.5)	0.008	0.018(4)	0.013
5.00	1.9	0.015(0.5)	0.010	0.026(2)	0.018

[a]Polarization measurements were carried out using the same spectrofluorimeter settings given in Table I except that the amplifier gain was 1.0–0.3. All samples were in phosphate buffer, pH 7.0, μ = 0.1 M and the temperature was 20 ± 1 °C. The Cu(I) samples were prepared by adding solid sodium ascorbate directly to the cuvette. [b]Viscosities were measured only for the Cu(II) solutions (data from ref. 12) and the Cu(I) solutions were assumed to have the same values. [c]Figures in brackets are the standard deviations in units of the last digit. [d]P_{corr} values were obtained by correcting the P_{obs} values to a viscosity of 1.0 cp.

10^{-5} M. P_0 did not vary systematically with concentration, so the average value of 0.100 ± 0.003 was used in the calculation below.

Emission Polarization of $Os(bpy)_3^{2+}$ in the Presence of Stellacyanin

Assuming a value of 0.100 for P_0, we calculated P_{max} for the probe bound to stellacyanin using eqn. (2). A value of 25 ns is the estimated average rotational relaxation time (ρ) for stellacyanin (eqns. (6) and (3)); hence, if the probe is fully bound to the protein, and if it is unable to undergo rotation independent of the protein, P_{max} would equal 0.031.

The observed emission polarization of $Os(bpy)_3^{2+}$ in the presence of Cu(I) and Cu(II) stellacyanin is given in Table II. Polarization values were adjusted to η = 1.0 cp (eqns. (2) and (3)) in order to distinguish between polarization changes due to increased interaction with the macromolecule and those due to the increased viscosity of the medium. Viscosities were measured for the Cu(II) stellacyanin solutions [12] only, and the viscosities of the Cu(I) solutions were assumed to vary in a similar manner.

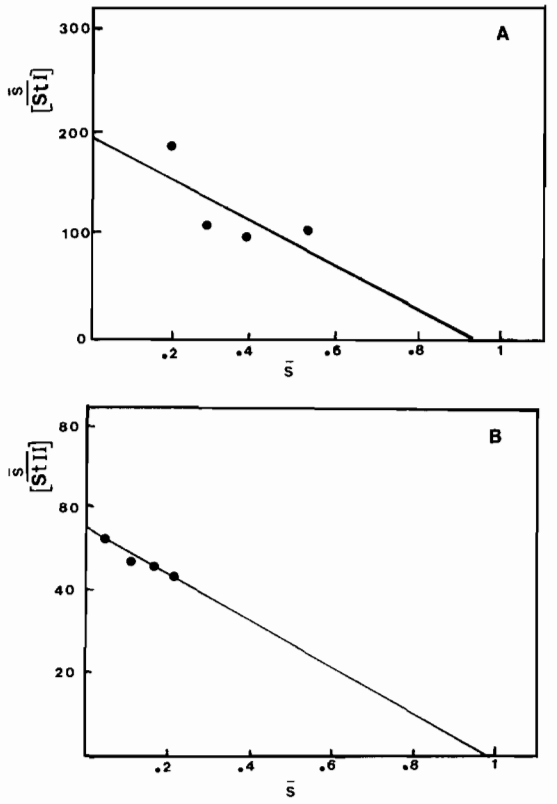

Fig. 2. Modified Scatchard plots of the binding of $Os(bpy)_3^{2+}$ to reduced (A) and oxidized (B) stellacyanin in phosphate buffer, pH 7.0, μ = 0.1 and 20 ± 1 °C. The fractional saturation of $Os(bpy)_3^{2+}$ ($\bar{s}$) was calculated assuming that P_{max} = 0.031 (see text).

The corrected data were fit to a modified Scatchard plot (eqn. (4)) with P_{max} = 0.031; [*St*] was assumed equal to the total protein concentration since in all cases the protein concentration was at least an order of magnitude greater than that of the probe. The modified Scatchard plots are shown in Fig. 2 for Cu(I) and Cu(II) stellacyanin, and $\bar{s}$, the fractional saturation of the ligand, *vs.* stellacyanin concentration is given in Table III using polarization data corrected to η = 1.0 cp.

Discussion

From the increase in the observed polarization with increasing stellacyanin concentration (Table II), we conclude that there is interaction between the protein and the probe at high protein concentration.

TABLE III. Fractional Saturation of $Os(bpy)_3^{2+}$ *vs.* Protein Concentration for Cu(I) and Cu(II) Stellacyanin[a]

[*St*] (mM)	Cu(I)		Cu(II)	
	P_{corr}[b]	$\bar{s}$	P_{corr}[b]	$\bar{s}$
0.00	0.001	0.000	0.0033	0.000
1.00	0.007	0.199	0.0049	0.058
2.50	0.010	0.299	0.0069	0.129
3.75	0.013	0.399	0.0085	0.187
5.00	0.018	0.565	0.0099	0.237

[a]The fractional saturation of $Os(bpy)_3^{2+}$, $\bar{s}$, is defined as the number of moles of ligand bound to stellacyanin per mole of ligand. The $\bar{s}$ values were determined assuming P_{max} = 0.031 and a 1:1 stoichiometry (see text). [b]Observed polarizations corrected to a viscosity of 1.0 cp.

If we assume that the bound probe does not undergo motion independent of the protein (*i.e.*, P_{max} = 0.031), association constants of 207 and 58 M^{-1} are calculated for the binding of $Os(bpy)_3^{2+}$ to Cu(I) and Cu(II) stellacyanin, respectively. In addition, the Scatchard plots indicate that the binding stoichiometry is 1:1 since extrapolation gives an intercept of ~1 in each case.

In the kinetic study on Cu(I) stellacyanin and $^*Ru(bpy)_3^{2+}$, half-saturation was observed at <1 mM protein [1]. The present analysis indicates that half-saturation should occur between 4–5 mM protein (Table III). An obvious explanation for this discrepancy is that $Os(bpy)_3^{2+}$ can undergo rotation independent of the protein. This would reduce P_{max} considerably from the calculated value of 0.031 and consequently increase the value determined for *K*. Also, the kinetic analysis of the $Ru(bpy)_3^{2+}$ reactions revealed that the data are best fit by a scheme which allows for the formation of a 2:1 complex at high protein concentration; *i.e.*, a complex in which the metal compound in 'sandwiched' between two protein molecules [1]. Such a scheme would also be consistent with the present data as long as the $Os(bpy)_3^{2+}$ can undergo rotation independent of the 'sandwich'. However, in this instance our Scatchard analysis is not valid since we assumed a 1:1 stoichiometry and also P_{max} would be greater than 0.031.

In addition to binding to the protein, another factor which would serve to increase the measured polarization is lifetime quenching by the protein. We neglected this in our calculations above since quenching rates similar to those observed in the $Ru(bpy)_3^{2+}$ experiments [1] would have a negligible effect on the measured polarization. Similarly, scattering by the protein solution would serve to increase the measured polarization. Again, contributions of scattering to the measured polarization are negligible since the excitation and emission wavelengths are well-separated and a cut-off filter was used for all measurements. Thus, in summary, the polarization data presented here corroborate the results of the quenching experiments: at high protein concentration the probe binds to the reduced protein and to a lesser extent to the oxidized protein (no saturation was observed up to 3 mM protein in the quenching of $^*Ru(bpy)_3^{2+}$ by Cu(II) stellacyanin, azurin or plastocyanin). However, during its excited state lifetime, the probe has considerable rotational freedom independent of the protein since the observed polarization is much less than the expected values for either a 1:1 or a 2:1 protein/probe complex.

Finally, it is of interest to note that the angle (α) between the absorption and emission transition dipoles for $Os(bpy)_3^{2+}$ can be obtained from P_0 since $P_0 = (3\cos^2\alpha - 1)/\cos^2\alpha + 3)$ [5]. For P_0 = 0.100, $\alpha = 48°$; *i.e.*, the absorption dipole for the transition centered at 480 nm, and the emission dipole (λ_{max} = 715 nm) are at 48° to each other. The absorption at 480 nm has been assigned to a metal-to-ligand charge transfer (MLCT) transition from a metal t_{2g} orbital to the lowest-lying π^* acceptor level on the bpy ligand [15]. Furthermore, the 480-nm band is primarily *xy*-polarized and therefore perpendicular to the C_3 axis of $Os(bpy)_3^{2+}$ which has D_3 symmetry. Hence, the emission dipole is at an angle of ~45° to the C_3 molecular axis.

Acknowledgements

This work was supported by a grant from Concordia University to A.M.E. Stellacyanin was a generous gift from Prof. H. B. Gray.

References

1 B. S. Brunschwig, P. J. Delaive, A. M. English, M. Goldberg, H. B. Gray, S. L. Mayo and N. Sutin, *Inorg. Chem.*, in press.
2 S. Wherland and H. B. Gray, in A. W. Addison, W. R. Cullen, D. Dolphin and B. R. James (eds.), 'Biological Aspects of Inorganic Chemistry', Wiley, New York, 1977, p. 289, and refs. therein; A. G. Mauk, R. A. Scott and H. B. Gray, *J. Am. Chem. Soc., 102*, 4360 (1980).
3 S. K. Chapman, I. Sanemasa, A. D. Watson and A. G. Sykes, *J. Chem. Soc., Dalton Trans.*, 1949 (1983) and refs. therein.
4 D. A. Deranleau and H. Neurath, *Biochemistry, 5*, 1413 (1966).
5 C. R. Cantor and P. R. Schimmel, in 'Biophysical Chemistry Part II: Techniques for the Study of Biological Structure and Function', Freeman, San Francisco, 1980, p. 454–465.
6 T. Omura, *J. Biochem. (Tokyo), 50*, 394 (1961).
7 D. J. Cookson, M. T. Hayes and P. E. Wright, *Biochim. Biophys. Acta, 591*, 162 (1980); D. J. Cookson, M. T.

Hayes and P. E. Wright, *Nature (London), 283*, 682 (1980).
8 P. M. Handford, H. A. O. Hill, R. W. K. Lee, R. A. Henderson and A. G. Sykes, *J. Inorg. Biochem., 13*, 83 (1980).
9 C. Cruetz, M. Chou, T. L. Netzel, M. Okumura and N. Sutin, *J. Am. Chem. Soc., 102*, 1309 (1980).
10 B. Reinhammar, *Biochim. Biophys. Acta, 205*, 35 (1970); B. G. Malmstrom, B. Reinhammar and T. Vanngard, *Biochim. Biophys. Acta, 205*, 48 (1970).
11 L. Stryer, *J. Mol. Biol., 13*, 482 (1965).
12 A. M. English, P. J. Delaive and H. B. Gray, unpublished results.
13 'CRC Handbook of Chemistry and Physics, 61st edn.', CRC Press, Fla., 1981, p. F–55.
14 C. R. Cantor and P. R. Schimmel, in 'Biophysical Chemistry Part II: Techniques for the Study of Biological Structure and Function', Freeman, San Francisco, 1980, p. 554.
15 A. B. P. Lever, in 'Inorganic Electronic Spectroscopy', 2nd edn., Elsevier, Amsterdam, 1984, p. 296–303, and refs. therein.