Pulse Radiolytic Measurement of Redox Potentials: The Tyrosine and Tryptophan Radicals†

Michael R. DeFelippis,‡ C. P. Murthy,‡ M. Faraggi,*,§ and Michael H. Klapper*,‡

Division of Biological Chemistry, Department of Chemistry, The Ohio State University, Columbus, Ohio 43210, and Department of Chemistry, Nuclear Research Centre—Negev, Beer-Sheva 84190, Israel

Received December 1, 1988; Revised Manuscript Received February 17, 1989

ABSTRACT: With the technique of pulse radiolysis we have measured the redox midpoint potentials of the tryptophan side chain neutral indolyl radical (1.05 ± 0.01 V vs NHE, pH 7.0 and 25 °C) and the tyrosine side chain neutral phenoxy radical (0.94 ± 0.01 vs NHE, pH 7.0 and 25 °C). These potentials were obtained by using a variety of inorganic reference compounds in both kinetic and equilibrium protocols. We compare these results with others already in the literature, and we also present data useful in establishing a pulse radiolysis redox reference scale over the range 0.42–1.28 V.

The normally short lived phenoxy radical of the tyrosine side chain (tyrO•)[1] is found as a stable species in a bacterial ribonucleotide reductase and is required for that enzyme's activity (Reichard & Ehrenberg, 1983). This same radical has been implicated recently in the oxidation of water by photosystem II (Barry & Babcock, 1987). Our interest in tyrO• stems from the observation in proteins and polypeptides (Prütz & Land, 1979; Prütz et al., 1980, 1981; Faraggi et al., 1989a) of the 1-electron intramolecular long-range electron transfers:

$$\text{trp}^{\bullet}\text{–tyrOH} \rightleftharpoons \text{trpH–tyrO}^{\bullet} \qquad (1)$$

$$\text{tyrOH–trp}^{\bullet} \rightleftharpoons \text{tyrO}^{\bullet}\text{–trpH}$$

In these reactions the tryptophan side chain neutral indolyl radical (trp•) oxidizes the tyrosine side chain phenol (tyrOH) to form tryptophan (trpH) and tyrO•. [In eq 1 we have indicated the reactions with the order of tyrosine and tryptophan reversed in the peptide, since we have found that the order can affect the electron-transfer rate (Faraggi et al., 1989a).] While this redox reaction is not known to occur naturally (and no role has yet been uncovered in nature for the tryptophan radical), it is one of a number of intramolecular electron transfers that have been recently studied in proteins, polypeptides, and other synthetic systems (Isied, 1984; Guarr & McLendon, 1985; Peterson-Kennedy et al., 1985; Mayo et al., 1986; Miller, 1987; McLendon, 1988). Moreover, the established ability of the tyrosine side chain to participate in 1-electron redox reactions and the importance of tyrO• in at least one enzymatic reaction and in photosynthesis suggest that this radical may function generally in biologically significant electron-transfer processes. We should also note that organic free radicals attached to proteins offer the potential of a rich new chemistry, although little attention has been paid them to date. There are numerous examples of protein-stabilized flavin [e.g., Massey and Hemmerich (1980)] and methoxatin [e.g., Kenney and McIntire (1983)] semiquinones, radicals produced upon the 1-electron reduction (oxidation) of quinones (hydroquinones). A protein-bound thiyl radical has been recently proposed (Knappe et al., 1984; Brush et al., 1988) as an intermediate in the pyruvate–formate lyase catalyzed pyruvate cleavage reaction.

An important property of any electroactive species is its standard redox potential, but for tyrO• and trp• these potentials are uncertain; the range of potentials reported for the tyrO•/tyrOH couple is 0.78–0.93 V (vs NHE) at pH 7 (Faraggi et al., 1988; Butler et al., 1986; Jovanovic et al., 1986; Harriman, 1987), and for trp•/trpH 0.64–1.08 V (Jovanovic et al., 1986; Faraggi et al., 1988; Butler et al., 1986; Harriman, 1987; Merényi et al., 1988). In an attempt to resolve the uncertainties, we have redetermined these redox potentials by the technique of pulse radiolysis. This technique permits generation and then observation of the tyrO• and trp• radicals in aqueous solution before they have had a chance to decay by radical–radical recombination or disproportionation reactions.

When short pulses (≤1 μs) of high-energy electrons (ca. 3.5 MeV from our accelerator) interact with water, the primary component of dilute aqueous solutions, the energy deposited results in the formation of the primary radicals $e_{aq}^{\bullet-}$, OH•, and H• (the last at much lower levels than the first two). We can convert $e_{aq}^{\bullet-}$ to OH• by reaction with saturating N_2O (reaction 2). The OH• formed in both the original pulse and reaction 2 reacts rapidly with the azide ion to produce the azide radical ($N_3^{\bullet}$) (reaction 3).

$$e_{aq}^{\bullet-} + N_2O + H_2O \rightarrow OH^{\bullet} + N_2 + OH^- \qquad (2)$$

$$OH^{\bullet} + N_3^- \rightarrow OH^- + N_3^{\bullet} \qquad (3)$$

The azide radical in turn is a good 1-electron oxidant of tyrosine and tryptophan, with the formation of trp• given as an example in eq 4. The tyrosine phenoxy radical and the

$$N_3^{\bullet} + \text{trpH} \rightarrow \text{trp}^{\bullet} + N_3^- + H^+ \qquad (4)$$

tryptophan indolyl radicals so produced are easily detected due to their strong absorbance bands at 405 and 510 nm, respectively (Bansal & Fessenden, 1976; Land & Prütz, 1979).

† This research was supported by NIH Grant GM-35718 and by Grant BSF 8500217 from the United States–Israel Binational Science Foundation (BSF), Jerusalem, Israel.

‡ The Ohio State University.

§ Nuclear Research Centre—Negev.

[1] Abbreviations: bpy, bipyridyl; $Br_2^{\bullet-}$, dibromide radical anion; $e_{aq}^{\bullet-}$, hydrated electron; OH•, hydroxyl radical; $N_3^{\bullet}$, azide radical; $(SCN)_2^{\bullet-}$, dithiocyanate radical anion; trpH, tryptophan; trp•, tryptophan with its side chain in the 1-electron oxidized neutral radical form; terpy, tripyridyl; $\text{trpH}^{\bullet+}$, tryptophan with its side chain in the oxidized radical cation form; tyrOH, tyrosine; tyrO•, tyrosine with its side chain in the 1-electron oxidized neutral radical form.

0006-2960/89/0428-4847$01.50/0

To obtain the redox potential of an unstable radical, such as trp•, we employ a second reference couple with a known 1-electron potential. From measurements of both the equilibrium position between radical and reference (reaction 5) and the forward and reverse rate constants with which that equilibrium is reached, we can obtain the reaction equilibrium constant from which we calculate the hitherto unknown radical redox potential. For unstable radicals, these measurements are possible provided the following conditions are met: (i) the reference system redox potential is near that of the radical under study; (ii) the radical and reference compounds are stable in both oxidation states for at least the time period required to reach equilibrium, a condition requiring that (iii) the electron transfer between radical and reference be fast relative to the radical's decay and (iv) the reference compound be soluble in water, the solvent in these experiments. Finally, because we monitor reactions from their absorbance changes, it is most convenient that (v) radical and reference in either or both of their oxidation states absorb (preferably in different regions) in the ultraviolet–visible.

$$H^+ + trp^\bullet + R \underset{k_b}{\overset{k_f}{\rightleftharpoons}} trpH + R^+ \qquad (5)$$

These conditions are generally not independent of one another. For example, the decays of both tyrO• and trp• are second order and so can be slowed by lowering the radical concentrations. Thus, in theory, we should be able to observe a slow reaction between radical and reference compound by going to ever lower radical concentrations; but the lowest practical radical concentration depends on the magnitude of the absorbance change that accompanies the redox reaction we hope to observe.

We have already reported 1-electron redox potentials of both tyrO•/tyrOH and trp•/trpH (Faraggi et al., 1988) obtained with reference ferrocene derivatives. However, the potentials of these ferrocenes were low relative to the potentials we had measured for the amino acid radicals, and we were uncertain as to the accuracy of those results. Hence, we have reinvestigated the reactions of tyrosine and tryptophan radicals with a variety of additional reference compounds, and we now report better estimates for the two redox potentials.

Materials and Methods

We synthesized $Os(bpy)_3Cl_2 \cdot 6H_2O$ by the procedure of Burstall et al. (1950) with only minor changes in extraction and purification steps. The visible absorption spectrum of our product was identical with that reported by these authors and the extinction coefficient of 13 300 M^{-1} cm^{-1} we obtained at 479 nm agreed with the 13 700 M^{-1} cm^{-1} at 477 nm reported in Schilt (1969). A proton NMR spectrum in D_2O was consistent with similar metal complexes of 2,2′-bipyridine and other group VIIIA metals (Lytle et al., 1971): 7.20 (triplet), 7.66 (doublet), 7.77 (triplet), and 8.43 (doublet) ppm. Anal. Calcd: C, 43.0; H, 4.33. Found: C, 41.4; H, 4.07.

We prepared $Os(terpy)_2Cl_2 \cdot 4H_2O$ by the method of Morgan and Burstall (1937), once again with minor changes in extraction and purification. The visible absorption spectrum in acetonitrile was in agreement with the one reported by Kober et al. (1985). High-resolution proton NMR in D_2O gave a spectrum consistent with peak assignments for $Os(terpy)_2^{2+}$ in Lytle et al. (1971): 6.94 (triplet), 7.16 (doublet), 7.63 (triplet), 7.80 (triplet), 8.35 (doublet), and 8.65 (doublet) ppm. Anal. Calcd: C, 45.0; H, 3.78. Found: C, 44.8; H, 3.90.

We based our preparation of K_2IrBr_6 on the procedure of Dwyer et al. (1947). To 1 g of $K_3Ir(NO_2)_6$ (K & K Laboratories, Cleveland, OH) were added 10 mL of bromine and 1 mL of HBr, and the solution was refluxed for 5 h. After evaporation of the reaction mixture, we added 2 mL of the bromine/HBr mixture, filtered off the undissolved solid, concentrated the filtrate to one-fifth of its original volume, and collected the shiny black crystalline solid, which was judged pure on the basis of its reported extinction coefficient at 584 nm (Melvin & Haim, 1977). To make K_3IrBr_6 we refluxed 0.8 g of K_2IrBr_6 refluxed in 50 mL of ethanol that also contained 10 mL of HBr for approximately 20 h. At this stage an equivalent amount of KBr was added and the mixture then refluxed for an additional 1 h. After cooling and concentration, we added ethanol to produce a green precipitate of K_3IrBr_6, which we collected by filtration and recrystallized from HBr by the slow addition of ethanol. The K_3IrBr_6 was characterized by its absorption spectrum both before and after its reoxidation with Br_2 and HBr (Melvin & Haim, 1977).

$Fe(bpy)_3(ClO_4)_2 \cdot H_2O$ was synthesized by the method of Dwyer and McKenzie (1947) and recrystallized twice from water. Its purity was established from its absorption spectrum (Szentrimay et al., 1977). As a check of its identity we compared its ^1H NMR spectrum with that of $Ru(bpy)_3^{2+}$ (Sadtler 25494M) and its ^{13}C NMR spectrum with that of bipyridine (Sadtler 19935C). In acetone the ^1H spectrum of the iron complex has peaks centered at 7.58 (triplet), 7.72 (doublet), 8.26 (triplet), and 8.83 (doublet) ppm. The ^{13}C spectrum, also taken in acetone, has peaks at 125.02, 128.58, 139.80, 155.19, and 160.30 ppm. $Ru(bpy)_3Cl_2 \cdot 6H_2O$ was purchased from Strem (Newburyport, MA) and used without further purification. Technical grade $NaClO_2$ was recrystallized three times from water and its purity determined on the basis of its absorbance at 262 and 292 nm (Eriksen et al., 1981). Na_3IrCl_6 was purchased from Alfa (Danvers, MA) and its purity established on the basis of its absorbance at 480 nm (ϵ = 4050; Poulsen & Garner, 1962) when dissolved in water.

All other chemicals were of the highest purity commercially available and were used as received. Water for the pulse radiolysis experiments was purified in a Millipore Milli Q apparatus.

The hydrolysis rate of the $IrCl_6^{3-}$ complex at pH 7, 0.05 M phosphate buffer, was measured by dissolving the sodium salt to 0.4 mM. Aliquots of 4.0 mL each were taken over a period of 7 h, and the complex was oxidized to $IrCl_6^{2-}$ with addition of 2.0 mL of chlorine water and 2.0 mL of concentrated perchloric acid. From the absorbance of the oxidized complex at 487 nm we could calculate the amount of $IrCl_6^{3-}$ remaining in the neutral aqueous solution. An estimated first-order rate constant was obtained by plotting the logarithm of the absorbance vs time.

We initiated radical reactions with a short (ca. 100 ns), high-energy electron (ca. 3.5 MeV from a Varian linear accelerator; Felix et al., 1967) pulse introduced into a 0.005 M, pH 7.0, phosphate buffer previously deaerated with oxygen-free N_2O (Meites & Meites, 1948). Dependent on the initiating inorganic radical desired, the solution also contained 0.05–0.1 M NaBr, KSCN, or NaN_3. The concentration, or pulse dose [determined by the method of Fielden (1982)], of the radicals $Br_2^{\bullet-}$, $(SCN)_2^{\bullet-}$, or $N_3^\bullet$ were normally kept between 0.5 and 2 μM to ensure pseudo-first-order kinetics, while the concentrations of all other solutes generally fell in the range 20 μM–20 mM. (The hexabromo- and hexachloroiridate solutions were used before hydrolysis became significant, within 20 min of their preparation.) To determine second-order rate constants for the oxidation of the various reference compounds (present in large excess) by the initiating inorganic radicals,

we varied the reference concentrations by 10-fold or greater. Reactions were monitored at a convenient wavelength (see Table I), and first-order rate constants were extracted from the time profiles with the nonlinear regression analysis provided in the data acquisition package ASYST (Macmillan Software, New York, NY), which does not provide estimated standard deviations. The rate constants so obtained were plotted against the concentration of the reference compound to obtain the second-order rate constant with a linear best fit.

The kinetic and equilibrium determinations of the K_{eq} between amino acid radical and reference compound were based on analysis of kinetic curves, such as that shown in the inset of Figure 1. The time profiles were treated with the ASYST nonlinear regression routine, and both k_{app} and ΔA_{eq} were extracted from the best fit. In the case of the kinetic protocol the dependence of these rate constants was then fit linearly to eq 6 by using the data analysis package MINSQ (Micromath, Salt Lake City, UT), which also provided us with estimated standard deviations for both the slope and intercept. With these values we obtained equilibrium constants, E_m, and, utilizing normal error propagation [e.g., Bevington (1969)], their associated standard deviations. For the equilibrium protocol we analyzed the dependence of ΔA_{eq} on reference compound concentration by fitting the data to either eq 12a, or 12b, or 12c as appropriate, with the nonlinear analysis routines of MINSQ. The standard deviation estimates for K_{eq} were propagated by standard techniques. It should be pointed out that since $\delta E'$ is computed from ln K_{eq}, relatively large standard deviations in K_{eq} result in small estimated standard deviations for $\delta E'$. In the many cases that we performed the kinetic and/or equilibrium protocols more than once, the standard deviations obtained upon averaging were ≤20 mV and were due entirely to reproducibility between sets of experiments rather than to the estimated standard deviations internal to each set. Our final midpoint potential values were obtained from unweighted averages as indicated in the text.

Differential pulse polarography experiments were performed with a Princeton Applied Research (Princeton, NJ) Model 173D potentiostat/galvanostat, a Model 179 Digital coulometer, and a Model 174A polarographic analyzer. These experiments were done in a Metrohm three-electrode glass cell (Brinkmann Instruments, Westbury, NY) using a glassy carbon working electrode of 0.2-cm^2 exposed area, platinum as a counter electrode, and calomel as the reference electrode. All experiments were performed with degassed solutions that were closely similar to those used in the pulse radiolysis experiments except for omission of the N_2O and replacement of the salt by KCl.

Results and Discussion

If radical and reference compounds react in the elementary (single step) reaction of eq 5, then there are two procedures by which to determine that reaction's equilibrium constant. The first is a kinetic method. With the initial concentration of the radical (e.g., trp$^\bullet$ in eq 5) much lower than the concentrations of both reference compound (R) and redox partner (in this example trpH), the redox reaction will be pseudo first order with a k_{app} given by

$$k_{app} = k_f[R] + k_b[trpH] \qquad [trp^\bullet] \ll [trpH], [R] \quad (6)$$

with k_f and k_b the forward and backward reaction rate constants, respectively. A plot of k_{app} versus the concentration of R at fixed trpH should then yield a straight line with intercept k_b[trpH] and slope k_f. Since [trpH] ≈ [trpH]$_0$ in our experiments, k_b can be calculated from the intercept and the equilibrium constant is obtained from the ratio k_f/k_b. (The reaction can also be studied at fixed reference and varied trpH concentrations with only a slight change in the analysis protocol.) From this equilibrium constant one obtains the potential difference, $\delta E'$ (eq 7) and then with eq 8 and the known reference redox potential, E', the redox potential of the unknown radical.[2]

$$\delta E' = (RT/nF) \ln K_{eq} \quad (7)$$

$$E'_{trp^\bullet/trpH} = \delta E' + E'_{R^+/R} \quad (8)$$

With this kinetic method and utilizing $Os(bpy)_3^{2+}$ as the reference compound R, we obtained a $\delta E'$ of 0.094 V for the equilibrium between trp$^\bullet$ and the osmium complex. From our electrochemically determined E' value for this organometallic, 0.83 V vs NHE, we estimated an E' of 0.92 V vs NHE for the trp$^\bullet$/trpH couple at 25 °C.

There is also a second, equilibrium, protocol with which to obtain redox potentials. At reaction equilibrium the final absorbance, A_{eq}, at any arbitrary wavelength is given (assuming a light path of 1 cm and reaction between trp$^\bullet$ and $Os(bpy)_3^{2+}$) by

$$A_{eq} = \epsilon_{Os(II)}[Os(II)]_{eq} + \epsilon_{trpH}[trpH]_{eq} + \epsilon_{Os(III)}[Os(III)]_{eq} + \epsilon_{trp^\bullet}[trp^\bullet]_{eq} \quad (9)$$

where $\epsilon_{Os(II)}$, $[Os(II)]_{eq}$, ϵ_{trpH}, $[trpH]_{eq}$, $\epsilon_{Os(III)}$, $[Os(III)]_{eq}$, $\epsilon_{trp^\bullet}$, and $[trp^\bullet]_{eq}$ are the extinction coefficients and equilibrium concentrations of $Os(bpy)_3^{2+}$, tryptophan, $Os(bpy)_3^{3+}$, and the tryptophan radical. Also needed for the analysis are expressions for the equilibrium constant, K_{eq}, and for the mass balance of each reaction species

$$K_{eq} = \frac{[Os(III)]_{eq}[trpH]_{eq}}{[Os(II)]_{eq}[trp^\bullet]_{eq}} \quad (10)$$

$$[Os(II)]_0 = [Os(II)]_{eq} + [Os(III)]_{eq}$$

$$[trpH]_0 = [trpH]_{eq} + [trp^\bullet]_{eq} \quad (11)$$

$$[trp^\bullet]_0 = [trp^\bullet]_{eq} + [Os(III)]_{eq}$$

where the subscript 0 refers to the initial concentration immediately after the pulse and before the equilibration between radical and reference has begun. For our experiments we measured the time-dependent absorbance changes at 535 nm, a wavelength that is near the trp$^\bullet$ absorbance maximum and where both $Os(bpy)_3^{2+}$ and $Os(bpy)_3^{3+}$, but not trpH, absorb. Once again the tryptophan and $Os(bpy)_3^{2+}$ concentrations were much greater than that of the pulse radiolytically formed trp$^\bullet$, and eq 9–11 simplify since

$$\epsilon_{trpH} = 0$$

$$[trpH]_0 \approx [trpH]_{eq}$$

$$[Os(II)]_0 \approx [Os(II)]_{eq}$$

Recognizing that the absorbance at time zero, A_0, is given by an equation similar to (9) in which the subscript eq is replaced by the subscript 0, we solve this set of equations by algebraic elimination to obtain

$$A_{eq} - A_0 = \frac{K_{eq}(\epsilon_{Os(III)} - \epsilon_{Os(II)}) + \epsilon_{trp^\bullet}[trpH]_0/[Os(II)]_0}{K_{eq} + [trpH]_0/[Os(II)]_0} \quad (12a)$$

[2] Note from eq 6 that the sign associated with the kinetically determined δE (eq 8) depends on which of the two initial concentrations is held constant and is not dependent on the direction the reaction takes toward equilibrium. However, the sign of δE is dependent on reaction direction in the case of the equilibrium protocol.

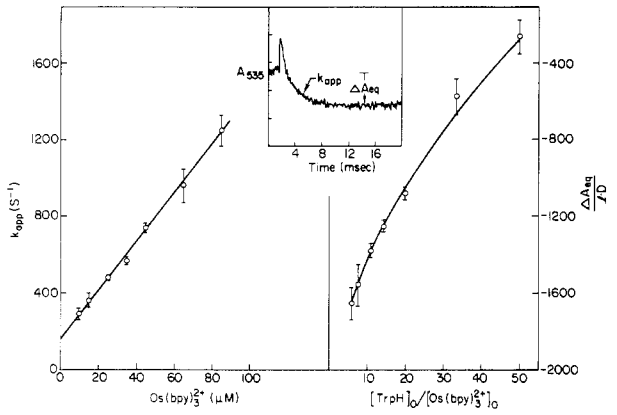

FIGURE 1: Equilibrium between the tryptophan side chain radical and $Os(bpy)_3^{2+}$. (Inset) Absorbance measured at 535 nm as a function of time. The apparent rate constant for the intramolecular electron transfer is obtained from the absorbance relaxation and the equilibrium position from the change of absorbance $A_\infty - A_0$ as indicated. The tryptophan, $Os(bpy)_3^{2+}$, and azide radical concentrations were 0.5 mM, 35 μM, and 1.24 μM, respectively. All other conditions are as described in the text. (Left) Plot of the apparent first-order rate constant as a linear function of the reference couple. The straight line drawn through the data points was obtained from a linear least-squares fitting. The intercept is taken as the fixed tryptophan concentration times the rate constant for the trpH oxidation by $Os(bpy)_3^{3+}$. The slope is the rate constant for the opposite oxidation, that of $Os(bpy)_3^{2+}$ by trp$^\bullet$. (Right) Plot of equilibrium absorbance (normalized with respect to the light path length, l, and the dose, D) as a function of the trpH/osmium complex concentration ratio. The curve was obtained by nonlinear least-squares fit of the data points to eq 12a. The equilibrium constants obtained by the two different techniques are discussed in the text.

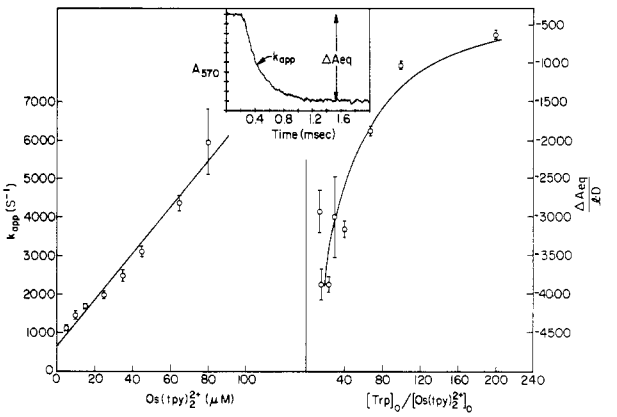

FIGURE 2: Equilibrium between the tryptophan side chain radical and $Os(terpy)_2^{2+}$. (Inset) Absorbance measured at 570 nm as a function of time. The apparent rate constant for the intermolecular electron transfer is obtained from the absorbance relaxation and the equilibrium position from the change of absorbance $A_\infty - A_0$ as indicated. The tryptophan, $Os(terpy)_2^{2+}$, and azide radical concentrations were 1 mM, 65 μM, and 0.8 μM, respectively. All other conditions are as described in the text. (Left) Plot of the apparent first-order rate constant as a linear function of the reference couple concentration. See the legend to Figure 1 for further explanation. (Right) Plot of equilibrium absorbance (normalized with respect to the light path length, l, and the dose, D) as a function of the trpH/osmium complex concentration ratio. The curve was obtained by nonlinear least-squares fit of the data points to eq 12b.

Since the three extinction coefficients are measured independently, the ratio of tryptophan to $Os(bpy)_3^{2+}$ is fixed by the known initial concentrations, and the trp$^\bullet$ concentration at time zero is calculated from the dose deposited by the electron pulse, we can evaluate K_{eq} from eq 12a by nonlinear analysis of the $A_{eq} - A_0$ dependence on the $[trpH]_0/[Os(bpy)_3^{2+}]_0$ ratio.

In the inset of Figure 1 we present the time profile of an example reaction between trp$^\bullet$ and $Os(bpy)_3^{2+}$. The initial rise in absorbance is due to the rapid $N_3^\bullet$ oxidation of trpH and formation of trp$^\bullet$. The subsequent slower absorbance decrease reflects equilibration between trp$^\bullet$ and the osmium complex. From the results of a series of such experiments at different tryptophan/osmium ratios and their best fit to the nonlinear eq 12a (Figure 1) we have extracted an equilibrium constant of 56 ± 2.6, a value that translates into a $\delta E'$ of 0.103 V. Once again using eq 8 and 0.83 V for the midpoint potential of the osmium complex, we obtain 0.93 V vs NHE as the trp$^\bullet$/trpH midpoint potential at 25 °C. This value compares well with the 0.92 V obtained by the kinetic method, as described earlier.

We believe, however, that a reliable estimate of the unknown potential may not be obtained when the reference and unknown potential difference is too large. Therefore, it is necessary to make comparisons with reference couples that span a voltage range into which the unknown is anticipated to fall. For this reason we used a number of additional references, among them $Os(terpy)_2^{3+}/Os(terpy)_2^{2+}$, with a midpoint potential of 0.93 V under our experimental conditions. The time profile of an example reaction between trp$^\bullet$ and $Os(terpy)_2^{2+}$ is shown in the inset of Figure 2. In this case there was no initial absorbance rise upon trpH oxidation since trp$^\bullet$ absorbs negligibly at 570 nm, the measurement wavelength. The absorbance decrease is due to the change that accompanies oxidation of the reduced osmium complex. As in the previous case, k_{app} increased linearly with increasing osmium complex concentration. From the slope and intercept of the plot in Figure 2 we obtained a midpoint potential of 1.06 V for the trp$^\bullet$/trpH couple. For the equilibrium protocol we modified eq 12a to reflect the negligible absorbance of trp$^\bullet$ at 570 nm.

$$A_{eq} - A_0 = \frac{K_{eq}(\epsilon_{Os(III)} - \epsilon_{Os(II)})}{K_{eq} + [trpH]_0/[Os(II)]_0} \qquad (12b)$$

The nonlinear least-squares fit (Figure 2) of the data to eq 12b yielded an equilibrium constant from which we obtained an E_m' of 1.03 V for the trp$^\bullet$/trpH couple. The results of the kinetic and equilibrium experiments once again agree well with one another. However, the midpoint potential obtained with $Os(terpy)_2^{2+}$ is not the same as that obtained with $Os(bpy)_3^{2+}$. We shall return shortly to this apparent discrepancy.

The final example is the measurement of the trp$^\bullet$/trpH redox couple with the reference $ClO_2^\bullet/ClO_2^-$. In this case only the neutral radicals, $ClO_2^\bullet$ and trp$^\bullet$, absorb, and the two absorbance bands were sufficiently different that we could measure either one or the other. Thus, when monitoring at 360 nm, the absorbance band of $ClO_2^\bullet$, we could use the following simplified version of eq 12a:

$$A_{eq} - A_0 = \frac{K_{eq}(\epsilon_{ClO_2^\bullet})}{K_{eq} + [trpH]_0/[ClO_2^-]_0} \qquad (12c)$$

The experimental results look similar to those obtained in Figures 1 and 2. The measured potentials for the trp$^\bullet$/trpH couple were 1.03 and 1.04 V by the kinetic and equilibrium protocols, respectively.

We have used a number of different reference couples, including the three examples just mentioned, to measure both the tyrO$^\bullet$/tyrOH and trp$^\bullet$/trpH potentials. To make these measurements, however, we needed the appropriate references. Previous work, our own and that of others, suggested that the two amino acid potentials we were seeking were >0.7 V, and so we looked for appropriate (on the basis of the five conditions given in the introduction) reference couples in the range 0.7–1.3 V. In Table I we list (from lowest to highest potential)

Table I: Properties of Reference Couples

reference	E_m' (V vs NHE) exp[b]	E_m' (V vs NHE) lit.	k_{oxid}[a] ($\times 10^8$ M^{-1} s^{-1}) $(SCN)_2^{\bullet-}$	$N_3^{\bullet}$	$Br_2^{\bullet-}$
ferrocene[c]					
(hydroxyethyl)-	0.42	0.40[d]	8.0 (625)	36 (625)	14 (625)
-1-carboxylate	0.53	0.53[d]	3.8 (625)	30 (625)	6.2 (625)
[*N,N'*-(dimethylamino)ethyl]-	0.59		3.6 (625)	30 (625)	6.0 (625)
-1,1'-dicarboxylate	0.66	0.65[d]	0.57 (625)	27 (625)	0.67 (625)
$IrBr_6^{3-}$	0.80	0.805[e]	1.9 (584)	18[f] (584)	6.8 (584)
$Os(bpy)_3^{2+}$	0.83	0.844[d]	27 (540)	>36 (540)	16 (360 and 535)
$IrCl_6^{3-}$	0.91	0.892[g]	0.049 (490)	4.6[f] (490)	0.15 (490)
$Os(terpy)_2^{2+}$	0.93	0.966[h]	15 (590)	44 (600)	18 (590)
ClO_2^-	0.92	0.936[i]	0.0012 (480)	0.78 (360)	0.0052 (360)
$Fe(bpy)_3^{2+}$	1.05	1.074 and 1.096[j]	2.1 (440)	29[f] (560)	1.3[k] (560)
$Ru(bpy)_3^{2+}$	1.28	1.27[d]	0.14 (480)	1.2 (500)	0.79 (360 and 480)

[a] Given in parentheses are wavelengths, in nanometers, at which the individual experiments were conducted. Rate constants were obtained under the conditions specified for radical oxidations of the reference compounds under Materials and Methods. [b] Measured electrochemically as described under Materials and Methods under conditions that were closely similar with those of the pulse radiolytic experiments; the only difference was the replacement of the salt (azide, thiocyanate, or bromide) used to generate the inorganic radical by equimolar Cl^-. [c] All the ferrocene data were reported previously in Faraggi et al. (1988). [d] Szentrimay et al. (1977). [e] Jackson and Pantony (1971). [f] Rate constants for the azide radical oxidation of $IrBr_6^{3-}$, $IrCl_6^{3-}$, and $Fe(bpy)_3^{2+}$ were reported to be 13×10^8, 5.5×10^8, and 15×10^8 M^{-1} s^{-1}, respectively, by Ram and Stanbury (1986). [g] Margerum et al. (1975). [h] Dwyer and Gyarfas (1954); approximately 0.1 M ionic strength in acid. [i] Three reports in the literature for potentials measured between pH 4 and 6: Troitskaya et al. (1958); Flis (1958); Merényi et al. (1988). [j] Dwyer and McKenzie (1947); Szentrimay et al. (1977). [k] Dimitrijević and Mičič (1982) have reported a rate constant of 1.0×10^8 M^{-1} s^{-1}.

those compounds we have found to date; included are the ferrocene derivatives from our earlier study (Faraggi et al., 1988). The reduced and oxidized partners of all these reference couples have different visible absorption spectra. (In general, we found the bi- and tripyridyl ligand containing organometallics difficult to use, because of their intense absorbances over most of the visible region.) We also include in Table I the wavelengths at which we performed the various experiments and the E_m' that we measured electrochemically at 25 °C under conditions similar to those of our pulse radiolysis experiments. All the oxidized species are stable within the millisecond time scale of our experiments, and all the reduced compounds are stable in aqueous solutions for more than 1 day, with two exceptions. Both $IrCl_6^{3-}$ and $IrBr_6^{3-}$ hydrolyze in aqueous solution. We have found by the technique described under Materials and Methods that the first half-life of the hexchloride hydrolysis is approximately 8 h. Thus, we prepared our solutions before each experiment and used no sample for longer than roughly 20 min. The hexabromide complex can be stabilized by addition of 0.1 M Br^- to the solution, which not only reverses the hydrolysis equilibrium but also serves as a precursor of the 1-electron $Br_2^{\bullet-}$ radical oxidant. Nonetheless, the hexabromide solutions were also used within approximately 20 min after we dissolved the $IrBr_6^{3-}$. Finally, we list in Table I the rate constants for the oxidation of each of the inorganic reference compounds by the azide, bromide, and thiocyanate radicals.[3] These rate constants are important since radical oxidation of reference versus sample compounds is competitive, and experimentation is easier if one can adjust initial reactant concentrations so as to produce predominantly either the reference or target radical.

In Table II we present the various tyrO$^{\bullet}$/tyrOH and trp$^{\bullet}$/trpH E_m' obtained by both kinetic (eq 8) and equilibrium (eq 9–12) protocols. The reader should notice that when both protocols were used with one reference we obtained similar, within experimental error, radical potentials. This establishes the validity of the assumption behind eq 5; namely, the reactions between amino acid radical and reference compound are single step. However, the measured trp$^{\bullet}$/trpH redox potential appears to increase with increasing reference couple potential and remains apparently constant only at or above 0.93 V. Clearly, a redox couple's E_m' should not depend on the potential of the reference used in its measurement. A reasonable, though not yet proven, conjecture in explanation of this apparent artifact is that the measured equilibrium constant becomes unworkably large (or small) at larger potential differences and so there is the introduction of systematic

Table II: Measured Redox Potentials of the Tryptophan and Tyrosine Radicals

reference	reference E_m'[b] (V)	radical E_m'[a] (V) kinetics	radical E_m'[a] (V) equilibrium	oxidant[c]
		Tryptophan		
ferrocene-1,1'-dicarboxylate$^{III/II}$	0.66	0.81	0.84	$N_3^{\bullet}$
$IrBr_6^{2-/3-}$	0.80	0.96	0.94	$Br_2^{\bullet-}$
$Os(bpy)_3^{3+/2+}$	0.83	0.92	0.93	$N_3^{\bullet}$
$IrCl_6^{2-/3-}$	0.91	1.02	1.02	$N_3^{\bullet}$ and $Br_2^{\bullet-}$
$ClO_2^{\bullet}/ClO_2^-$	0.92	1.03	1.04	$N_3^{\bullet}$ and $Br_2^{\bullet-}$
$Os(terpy)_2^{3+/2+}$	0.93	1.06	1.03	$N_3^{\bullet}$
$NO_2^{\bullet}/NO_2^-$	1.03[d]	1.07	–	$Br_2^{\bullet-}$
$Fe(bpy)_3^{3+/2+}$	1.05	1.09	1.00	$N_3^{\bullet}$
		Tyrosine		
ferrocene-1,1'-dicarboxylate$^{III/II}$	0.66	>0.8	–	$N_3^{\bullet}$
$IrBr_6^{2-/3-}$	0.80	0.92	0.92	$Br_2^{\bullet-}$
$Os(bpy)_3^{3+/2+}$	0.83	0.97	0.94	$N_3^{\bullet}$
$IrCl_6^{2-/3-}$	0.91	0.94	0.96	$Br_2^{\bullet-}$ and $N_3^{\bullet}$
$ClO_2^{\bullet}/ClO_2^-$	0.92	0.94	–	$Br_2^{\bullet-}$
$Os(terpy)_2^{3+/2+}$	0.93	–	0.90	$N_3^{\bullet}$

[a] pH 7.0, 25 °C, versus NHE. The description of the kinetic and equilibrium procedures is given in the text. The estimated standard deviation associated with each E_m' is ≤20 mV. [b] Taken from our experimental results of Table I, unless otherwise noted. [c] The radical produced with in the first microsecond that is then used to oxidize the amino acid or inorganic reference compound. When two radicals are indicated, both were used in separate experiments. [d] Wilmarth et al. (1983).

[3] The rate constants for tyrOH oxidation by $(SCN)_2^{\bullet-}$, $N_3^{\bullet}$, and $Br_2^{\bullet-}$ are 5.0×10^6, 1.0×10^8, and 2.0×10^7 M^{-1} s^{-1}, respectively, and compare well with rate constants in the literature (Adams et al., 1972; Land & Prütz, 1979; Jovanovic et al., 1986). Similarly for trpH oxidation the three rate constants are 3×10^8, 3.0×10^9, and 7.1×10^8 M^{-1} s^{-1}. It is interesting to note that while the redox potentials of $(SCN)_2^{\bullet-}/2SCN^-$, $N_3^{\bullet}/N_3^-$, and $Br_2^{\bullet-}/2Br^-$ are 1.29, 1.33, and 1.63 V, respectively (DeFelippis, Klapper, and Faraggi, unpublished data; Mamou et al., 1977), the $N_3^{\bullet}$ oxidation is the fastest of the three for each of the compounds mentioned in this paper and is at or near the diffusion-controlled limit (Faraggi et al., 1988) for each of the reference compounds.

error(s). We have observed a similar "drift" in other work (McWhirter and Klapper, unpublished data), and the results reported here highlight the requirement mentioned in the introduction—the redox potentials of reference and unknown should be as close as possible to one another to ensure a reasonable determination of the unknown potential. The practical consequence is that with little known about the value of a radical's redox potential, one should verify any single result using an additional reference couple with a potential presumably closer to that of the radical. Having considered this precaution, we calculated the tyrO•/tyrOH midpoint potential as the unweighted average of the values obtained with the four reference compounds $Os(bpy)_3^{2+}$, $IrCl_6^{3-}$, ClO_2^-, and $Os(terpy)_2^{2+}$ and the trp•/trpH midpoint potential as the average of the values obtained with ClO_2^-, $Os(terpy)_2^{2+}$, NO_2^-, and $Fe(bpy)_3^{2+}$. The calculated tyrO•/tyrOH and trp•/trpH midpoint potentials are 0.94 ± 0.01 and 1.05 ± 0.01 V, respectively, both vs NHE and at 25 °C and pH 7.0.

The calculated difference in these two potentials, 110 ± 15 mV, indicates that the equilibrium reaction

$$\text{trp}^\bullet + \text{tyrOH} \rightleftharpoons \text{trpH} + \text{tyrO}^\bullet \qquad (13)$$

lies toward the right. Thus, there is an independent measurement, a test of internal consistency, for this calculated potential difference—direct determination of the equilibrium constant for the reaction 13 intermolecular oxidation of tyrOH by trp•. This was, however, a difficult measurement since the electron transfer is slow (k_f is ca. 1×10^5 M^{-1} s^{-1}) and the tyrosine solubility is low (ca. 2 mM under our experimental conditions). Nonetheless, we were able with the equilibrium protocol to obtain an approximate $\delta E'$ of 80 ± 10 mV, a value close to the calculated 110 mV.

Since there have been such wide variations in the literature values of both tyrO•/tyrOH and trp•/trpH redox potentials, it pays to compare our present data with the various other results. The very low trp•/trpH value of 0.64 V reported by Jovanovic and co-workers (Jovanovic et al., 1986) must be incorrect. Not only is it sharply different from estimates from all other laboratories, but it is also inconsistent with the remaining data in the very same paper, as pointed out by Butler et al. (1987). Thus, from the redox potential Jovanovic et al. reported for the trp•/trpH couple at pH 13, we can calculate an E_m' of 0.95 V, a value approximately 100 mV below ours. These authors also report a tyrO•/tyrOH E_m' of 0.85 V, again approximately 100 mV lower than ours. Thus, their results appear similar to our own, but with both redox potentials approximately 100 mV more negative. Our own earlier published estimates (Faraggi et al., 1988) for both amino acid couples were also incorrect, as already discussed above. Butler and co-workers (Butler et al., 1986) reported a trp•/trpH potential of 0.87 V in the peptide trpH-tyrOH. But this value was calculated by using an estimated and not experimentally determined tyrO•/tyrOH reference potential of 0.60 V at pH 13. Of significance is their experimentally determined $\delta E'$ of 93 mV between the tyrosine and tryptophan potentials, close to our own 110 mV. The tyrosine and tryptophan E_m''s that Harriman (1987) obtained by cyclic voltammetry are 0.93 and 1.015 V, respectively, close to the values we found by differential pulsed polarography (DPP), 0.93 and 1.02 V (Faraggi et al., 1989b). Although theoretically expected (Andrieux et al., 1970), we were pleasantly surprised that redox potentials obtained from irreversible electrochemical processes are so close to those measured in our pulse radiolysis equilibrium experiments. Since cyclic voltammetry and DPP are simpler, faster, and cheaper techniques than pulse radiolysis, they should be useful in future experiments with those radicals that dimerize rapidly to electrochemically inactive species. Finally, Merényi et al. (1988) have reported the value of 1.08 ± 0.02 V, obtained with the kinetic protocol and $ClO_2^•/ClO_2^-$ as reference—a result similar to our own within experimental error.

We propose that the uncertainties surrounding the redox potentials of both the tyrosine and tryptophan neutral radical species at pH 7 are now settled and that the values of 0.94 and 1.05 V, respectively, are good estimates within a range of no more than ±0.02 V. We have evidence, however, that when these two amino acid radicals are incorporated into peptides, their redox potentials change (Faraggi et al., 1989b). We are currently attempting to quantify these changes.

Registry No. TyrO•, 16978-66-8; Trp•, 100927-20-6; K_2IrBr_6, 19121-78-9; $K_3Ir(NO_2)_6$, 38930-18-6; K_3IrBr_6, 28235-14-5.

References

Adams, G. E., Aldrich, J. E., Bisby, R. H., Cundall, R. B., Redpath, J. L., & Willson, R. L. (1972) *Radiat. Res. 49*, 278–289.

Andrieux, C. P., Nadjo, L., & Saveant, J. M. (1970) *J. Electroanal. Chem. Interfacial Electrochem. 26*, 147–186.

Bansal, K. M., & Fessenden, R. W. (1976) *Radiat. Res. 67*, 1–8.

Barry, B. A., & Babcock, G. T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7099–7103.

Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, McGraw-Hill, New York.

Brush, E. J., Lipsett, K. A., & Kozarich, J. W. (1988) *Biochemistry 27*, 2217–2222.

Burstall, F. H., Dwyer, F. P., & Gyarfas, E. C. (1950) *J. Chem. Soc.*, 953–955.

Butler, J., Land, E. J., Prütz, W. A., & Swallow, A. J. (1986) *J. Chem. Soc., Chem. Commun.* 348–349.

Butler, J., Land, E. J., & Swallow, A. J. (1987) *J. Phys. Chem. 91*, 3114–3116.

Dimitrijević, N. M., & Micić, O. I. (1982) *J. Chem. Soc., Dalton Trans.*, 1953–1957.

Dwyer, F. P., & McKenzie, H. A. (1947) *J. Proc. R. Soc. N.S.W. 81*, 93–96.

Dwyer, F. P., & Gyarfas, E. C. (1954) *J. Am. Chem. Soc. 76*, 6320–6321.

Dwyer, F. P., McKenzie, H. A., & Nyholm, R. S. (1947) *J. Proc. R. Soc. N.S.W. 81*, 216–220.

Eriksen, T. E., Lind, J., & Merényi, G. (1981) *J. Chem. Soc., Faraday Trans. 1 77*, 2115–2123.

Faraggi, M., Weinraub, D., Broitman, F., DeFelippis, M. R., & Klapper, M. H. (1988) *Radiat. Phys. Chem. 32*, 293–297.

Faraggi, M., DeFelippis, M. R., & Klapper, M. H. (1989a) *J. Am. Chem. Soc.* (in press).

Faraggi, M., Broitman, F., DeFelippis, M. R., Weinraub, D., & Klapper, M. H. (1989b) *J. Phys. Chem.* (submitted for publication).

Felix, W. D., Gall, B. L., & Dorfman, L. M. (1967) *J. Phys. Chem. 71*, 384–392.

Fielden, E. M. (1982) in *The Study of Fast Processes and Transient Species by Electron Pulse Radiolysis* (Baxendale, J. H., & Busi, F., Eds.) pp 49–62, Reidel, Dordrecht, The Netherlands.

Flis, I. E. (1958) *Zh. Fiz. Khim. 32*, 573–579.

Guarr, T., & McLendon, G. (1985) *Coord. Chem. Rev. 68*, 1–52.

Harriman, A. (1987) *J. Phys. Chem. 91*, 6102–6104.

Hush, N. S. (1985) *Coord. Chem. Rev. 64*, 135–157.

Isied, S. S. (1984) *Prog. Inorg. Chem. 32*, 443–517.

Jackson, E., & Pantony, D. A. (1971) *J. Appl. Electrochem. 1*, 113–126.
Jovanovic, S. V., Harriman, A., & Simic, M. G. (1986) *J. Phys. Chem. 90*, 1935–1939.
Kenney, W. C., & McIntire, W. (1983) *Biochemistry 22*, 3858–3868.
Knappe, J., Neugebauer, F. A., Blaschkowski, H. P., & Gänzler, M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1332–1335.
Kober, E. M., Marshall, J. L., Dressick, W. J., Sullivan, B. P., Caspar, J. V., & Meyer, T. J. (1985) *Inorg. Chem. 24*, 2755–2763.
Land, E. J., & Prütz, W. A. (1979) *Int. J. Radiat. Biol. Relat. Stud. 36*, 75–83.
Lytle, F. E., Petrosky, L. M., & Carlson, L. R. (1971) *Anal. Chim. Acta 57*, 239–247.
Mamou, A., Rabani, J., & Behar, D. (1977) *J. Phys. Chem. 81*, 1447–1448.
Margerum, D. W., Chellappa, K. L., Bossu, F. P., & Burce, G. L. (1975) *J. Am. Chem. Soc. 97*, 6894–6896.
Massey, V., & Hemmerich, P. (1980) *Biochem. Soc. Trans. 8*, 246–257.
Mayo, S. L., Ellis, W. R., Crutchley, R. J., & Gray, H. B. (1986) *Science 233*, 948–952.
McLendon, G. (1988) *Acc. Chem. Res. 21*, 160–167.
Meites, L., & Meites, T. (1948) *Anal. Chem. 20*, 984–985.
Melvin, W. S., & Haim, A. (1977) *Inorg. Chem. 16*, 2016–2020.
Merényi, G., Lind, J., & Shen, X. (1988) *J. Phys. Chem. 92*, 134–137.
Miller, J. R. (1987) *Nouv. J. Chim. 11*, 83–89.
Morgan, G., & Burstall, F. H. (1937) *J. Chem. Soc.*, 1649–1655.
Peterson-Kennedy, S. E., McGourty, J. L., Ho, P. S., Sutoris, C. J., Liang, N., Zemel, H., Blough, N. V., Margoliash, E., & Hoffman, B. M. (1985) *Coord. Chem. Rev. 64*, 125–133.
Poulsen, I. A., & Garner, C. S. (1962) *J. Am. Chem. Soc. 84*, 2032–2037.
Prütz, W. A., & Land, E. J. (1979) *Int. J. Radiat. Biol. 36*, 513–520.
Prütz, W. A., Butler, J., Land, E. J., & Swallow, A. J. (1980) *Biochem. Biophys. Res. Commun. 96*, 408–414.
Prütz, W. A., Land, E. J., & Sloper, R. W. (1981) *J. Chem. Soc., Faraday Trans. 1 77*, 281–292.
Ram, M. S., & Stanbury, D. M. (1986) *J. Phys. Chem. 90*, 3691–3696.
Reichard, P., & Ehrenberg, A. (1983) *Science 221*, 514–519.
Schilt, A. A. (1969) in *Analytical Applications of 1,10-Phenanthroline and Related Compounds* (Belcher, R., & Freiser, H., Eds.) Vol. 32, International Series of Monographs in Analytical Chemistry, Pergamon Press, Oxford, U.K.
Szentrimay, R., Yeh, P., & Kuwana, T. (1977) in *Electrochemical Studies of Biological Systems* (Sawyer, D. T., Ed.) ACS Symposium Series 38, pp 143–169, American Chemical Society, Washington, DC.
Troitskaya, N. V., Mishchenko, K. P., & Flis, I. E. (1959) *Russ. J. Phys. Chem. 33*, 77–79.
Wilmarth, W. K., Stanbury, D. M., Byrd, J. E., Po, H. N., & Chua, C. P. (1983) *Coord. Chem. Rev. 51*, 155–179.

Solution Conformation of Conotoxin GI Determined by ^{1}H Nuclear Magnetic Resonance Spectroscopy and Distance Geometry Calculations

Yuji Kobayashi,*,‡ Tadayasu Ohkubo,‡,§ Yoshimasa Kyogoku,‡ Yuji Nishiuchi,∥ Shumpei Sakakibara,∥ Werner Braun,⊥,○ and Nobuhiro Gō○,△

Institute for Protein Research, Osaka University, Suita, Osaka 565, Japan, Peptide Institute Inc., Protein Research Foundation, Ina, Minoh, Osaka 562, Japan, and Department of Physics, Faculty of Science, Kyushu University, Kyushu 812, Japan

Received November 30, 1988; Revised Manuscript Received February 8, 1989

ABSTRACT: Conformational analysis of conotoxin GI, one of the neurotoxic peptides produced by a marine snail, genus *Conus*, was performed by a combination of nuclear magnetic resonance spectroscopy (NMR) and distance geometry calculations. The resulting conformers on minimization of the target function were classified into two groups. The difference in the structures of the conformers is mainly due to the difference in the orientation of the side chain of the tyrosyl residue. The results show that the solution structure of conotoxin GI satisfies the conformational requirements for the biological activity of an antagonist toward nicotinic cholinergic receptors elucidated in a series of studies on alkaloids. The structure is discussed on the basis of the results of comparison of the atomic arrangements of the active sites of snake venom peptides and molecular models based on the results of secondary structure prediction.

Fish-eating marine snails of the genus *Conus* produce several classes of toxic peptides that specifically bind to key elements in nerve and muscle cell membranes, resulting in successive blocking of the series of neuromuscular systems of fish (Kohn et al., 1960; Endean & Rudkin, 1963; Spence et al., 1977; Cruz et al., 1978; Olivera et al., 1985). They are called conotoxins and are classified into three large groups: α-conotoxins are presynaptic (Gray et al., 1984; McIntosh et al., 1982), ω-conotoxins are postsynaptic (Olivera et al., 1984, 1987), and μ-conotoxins are muscle channel inhibitors (Cruz et al., 1985). Gray et al. have systematically studied these peptides and have shown that commonly they are relatively small peptides of 13–29 amino acid residues and include 2–3 disulfide bonds.

* Author to whom correspondence should be addressed.
‡ Osaka University.
∥ Present address: Protein Engineering Research Institute, Suita, Osaka 565, Japan.
∥ Peptide Institute Inc.
⊥ Present address: Institute fur Molekularbiologie u. Biophysik, ETH, CH-8093 Zurich, Switzerland.
○ Kyushu University.
△ Present address: Department of Physics, Faculty of Science, Kyoto University, Kyoto 606, Japan.

0006-2960/89/0428-4853$01.50/0