Bovine Neurophysin I Dimerization Studied by Rapid Kinetic Techniques†

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ABSTRACT: Kinetic studies of the dimerization of bovine neurophysin I have been carried out by using temperature-jump and stopped-flow spectrometry. The reaction was monitored with the indicator bromophenol blue, which was shown to preferentially bind the monomeric form of neurophysin I, $K_B = 5.36 \times 10^4$ M^{-1}. Although most solutions showed a single relaxation time in the millisecond time region, a few solutions exhibited two times. Both relaxation times were strongly dependent on neurophysin concentration and only slightly dependent on bromophenol blue concentration. The faster relaxation time showed a marked pH dependence. Both protein dimerization and dye binding were included in the two-step mechanism which was found to fit the data. The rate constants for protein association and dissociation were 9.2×10^4 M^{-1} s^{-1} and 12 s^{-1}, respectively. The rate constants for neurophysin monomer binding to bromophenol blue were 1.1×10^6 M^{-1} s^{-1} and 20 s^{-1} for the association and dissociation, respectively. The ratios of rate constants for dimerization and indicator binding were consistent with the independently determined equilibrium association constants. Neurophysin dimerization occurs with rate constants comparable to those which have been found for other dimerizing protein systems. Bromophenol blue is postulated to form an ion-pair complex with neurophysin I which is stabilized by hydrogen-bond formation. Our results are compatible with a pH-independent dimerization of neurophysin I and imply that dimer formation depends upon hydrophobic interactions between the monomer subunits.

Neurophysins, a class of proteins which are found in the posterior pituitary gland, form noncovalent complexes with the hormones oxytocin and vasopressin [for a comprehensive review see Walter (1975)]. This system has been utilized as a model with which to study protein–hormone interactions. Bovine neurophysins I and II, which differ slightly in amino acid composition, are the major neurophysin species found in the cow (Walter et al., 1971; North et al., 1975; Wuu & Crumm, 1976). Thermodynamic studies have indicated that neurophysin dimerizes (Breslow et al., 1971; Nicolas et al., 1976) and that the dimer binds hormone more strongly than the monomer (Nicolas et al., 1976, 1978). This has been confirmed by kinetic studies of the binding reaction which showed that formation rate constants for the interaction of neurophysin dimer with protonated hormone were approximately 1 order of magnitude faster than those for the monomer (Pearlmutter & McMains, 1977). As part of a continuing investigation of the dynamics of neurophysin hormone interactions, I present here the first rapid kinetic study of the dimerization of bovine neurophysin I.

An initial screening with a series of pH indicators showed no observable pH-dependent kinetic effect. Therefore, bromophenol blue, which is known to bind to neurophysin (Burford & Pickering, 1972), was used as a probe to follow the dimerization reaction.

Materials and Methods

Bovine neurophysin I was prepared from a posterior pituitary acetone-desiccated powder (Pel-Freez) as described previously (Pearlmutter & McMains, 1977). Mononitrated bovine neurophysin I was prepared according to the procedure of Breslow & Gargiulo (1977). All neurophysin was stored as a solid in a vacuum desiccator at 4 °C.

Bromophenol blue was purchased from Kodak and dissolved in 1% ethanol to a concentration of 1 mM. This stock solution was then utilized for all further experiments.

Kinetic measurements were made in a Gibson-Durrum stopped-flow temperature-jump spectrophotometer at $I = 0.1$ M KCl and 25 °C. The magnitude of the temperature jump was determined as previously described (Pearlmutter & McMains, 1977), and the initial temperature was maintained at 9 °C so that after a 5-kV discharge through the cuvette the final temperature was 25 °C. In some cases, relaxation measurements were obtained from stopped-flow experiments where the data at close to equilibrium were treated as concentration perturbations (Pearlmutter & Stuehr, 1968).

Relaxation times were determined from at least three oscilloscope tracings by enlarging the Polaroid photographs, plotting log amplitude vs. time, and determining the half-times. Half-times were converted to relaxation times with the conversion factor $\tau = t_{1/2}/\ln 2$ (Eigen & DeMaeyer, 1963).

The magnitude of the binding constant for the interaction of bromophenol blue and bovine neurophysin I was determined spectrophotometrically by measuring the decrease in absorbance at 442 nm upon mixing the two components. All spectral measurements were made in a potassium phthalate-HCl buffer at $I = 0.1$, pH 3.8.

Results

The spectra of bromophenol blue (1.2×10^{-5} M) and a mixture of bromophenol blue (1.2×10^{-5} M) and bovine neurophysin I (6.7×10^{-5} M) were examined in the visible region of the spectrum, 400–650 nm, at $I = 0.1$, pH 3.8, and 25 °C (Figure 1). With bromophenol blue alone, peaks appeared at 440 and 598 nm, which could be assigned to the yellow or acidic form, HIn^-, and the purple or basic form, In^{2-}, respectively (Brode, 1924). Absorbance changes at both 440 and 598 nm followed Beer's law at bromophenol blue concentrations between 1.23×10^{-5} and 3.08×10^{-6} M. Two other pH indicators, methyl orange ($pK = 3.90$) and *m*-cresol purple ($pK = 1.51$), showed no difference spectra when mixed with neurophysin I.

In order to determine the magnitude and stoichiometry of the binding of the dye to neurophysin I, spectra were taken of a series of solutions containing bromophenol blue [typically $(1–2) \times 10^{-5}$ M] and varying amounts of neurophysin I [$(0.05–2.00) \times 10^{-4}$ M] on the Gilford spectrophotometer. The

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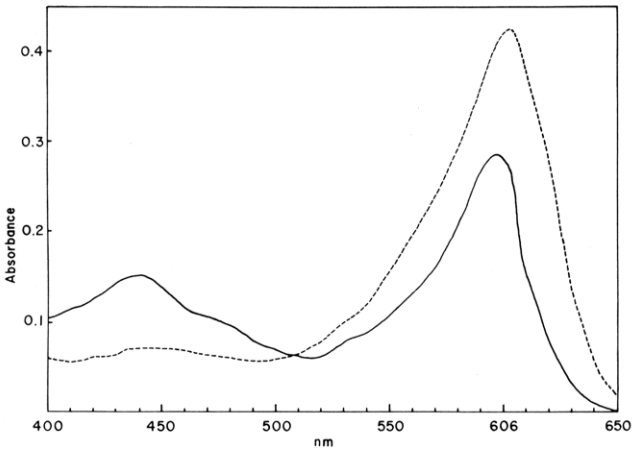

FIGURE 1: Absorption spectra of bromophenol blue, 1.2×10^{-5} M (—), and of a mixture of bromophenol blue, 1.2×10^{-5} M, and bovine neurophysin I, 6.7×10^{-5} M (---). Spectra were obtained in a Gilford spectrophotometer at 25 °C, $I = 0.1$ M KCl, and pH 3.8 in a potassium phthalate-HCl buffer.

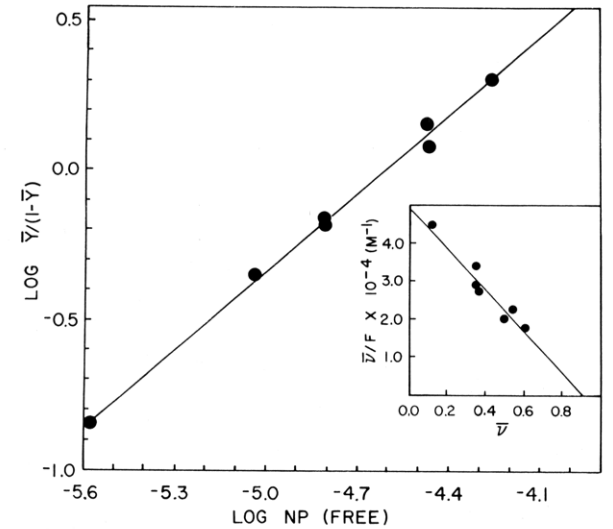

FIGURE 2: A Hill plot of the binding of bromophenol blue to bovine neurophysin I. $\bar{Y}$ represents the fractional saturation of bromophenol blue as stated in the text. The slope of the line is 0.87 with a correlation coefficient of 0.998. The inset shows the binding data plotted according to Scatchard where $\bar{\nu} = [\text{NPH}\cdots\text{In}]/[\text{HIn}^-]$ and F is [NP], unbound neurophysin I monomer.

spectrum of the dye–protein complex was independent of the protein to dye ratio. The percent decrease in absorbance at 442 nm with increasing neurophysin concentration was attributed to the formation of a neurophysin–bromophenol blue complex. The data were analyzed in terms of a Hill plot (Figure 2) with respect to neurophysin I concentration where $\bar{Y}$ is the fractional saturation of bromophenol blue and (Levitzki & Schlesinger, 1974)

$$\frac{\bar{Y}}{1-\bar{Y}} = \frac{[\text{bound dye}]}{[\text{bound dye}]_{max} - [\text{bound dye}]} \quad (1)$$

Examination of the binding data in this form gives a Hill coefficient, n, of 0.87 which indicates that dye binding to the protein shows negative cooperativity with respect to the protein dimerization reaction (Levitzki & Schlessinger, 1974). Thus, binding of bromophenol blue to the protein enhances the dissociation of neurophysin I dimer. The data were then plotted according to Scatchard (1949) (Figure 2 inset). With the use of the known dimerization constant of bovine neurophysin I, 7.7×10^3 M^{-1} (Nicolas et al., 1976), fractional

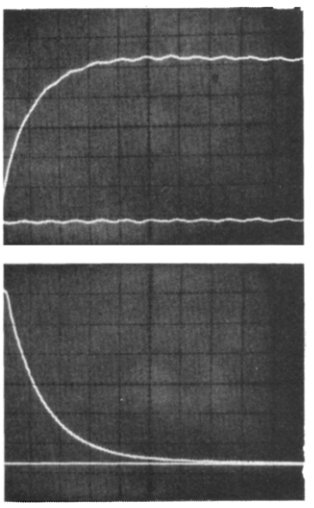

FIGURE 3: Typical temperature-jump (top) and stopped-flow (bottom) kinetic traces for the neurophysin I–bromophenol blue system. For the temperature-jump trace, $[\text{NPI}]^0 = 6 \times 10^{-5}$ M and $[\text{BPB}]^0 = 5 \times 10^{-5}$ M at pH 3.12 in 0.1 M KCl. Time scale is 20 ms per horizontal division, and the vertical scale is 200 mV per division. The reaction was monitored at 590 nm, and a 5-kV discharge through the cuvette resulted in a final temperature of 25 °C. The relaxation time for this solution was 19.6 ms. For the stopped-flow trace, $[\text{NPI}]^0 = 1.08 \times 10^{-4}$ M and $[\text{BPB}]^0 = 1 \times 10^{-5}$ M at pH 3.78 in 0.1 M KCl. Time scale is 20 ms per horizontal division, and the vertical scale is 500 mV per division. The reaction was monitored at 620 nm at 25 °C. The relaxation time, calculated for the last third of the reaction, was 26 ms.

saturation ratios, $\bar{\nu}$, were expressed as either bound ligand per mole of neurophysin monomer (Figure 2 inset) or bound ligand per mole of neurophysin dimer. With the assumption that only the monomeric form of the protein binds the dye, a straight line was obtained with a slope of $K_B = 5.36 \times 10^4$ M^{-1} (Figure 2 inset). If it is assumed that the dimer is the reactive species, a negative binding constant which does not have physical significance is obtained. The intercept on the x axis indicates that 1 mol of bromophenol blue binds about 0.9 mol of neurophysin monomer. This corresponds to an approximately 1:1 stoichiometry between dye and neurophysin monomer under the experimental conditions utilized. Identical spectra were obtained with mononitrated neurophysin I.

On the basis of the spectral and equilibrium binding experiments, I concluded that at pH 3.8 the most significant binding interaction between the dye and the protein involved the formation of a 1:1 complex between the bromophenol blue and the neurophysin monomer, with K_B of 5.36×10^4 M^{-1}.

For the temperature-jump experiments, known amounts of bromophenol blue and neurophysin I were dissolved in degassed 0.1 M KCl, and the pH was adjusted by using KOH and HCl. For the stopped-flow experiments, one syringe was filled with bromophenol blue and the other was filled with neurophysin I; both solutions were at the same initial pH and ionic strength. The final pH was determined by mixing equal aliquots of the two solutions. All relaxation spectra were monitored at 590 nm, corresponding to the absorption maximum of bromophenol blue.

In the presence of either bromophenol blue alone, bovine neurophysin I alone, or nitrated bovine neurophysin I alone, no relaxation effect was seen. When either methyl orange or *m*-cresol purple was mixed with neurophysin I, no relaxation effect was seen. When a solution containing both bromophenol blue and neurophysin I was examined, a relaxation effect in the millisecond time region was observed. Typical kinetic data obtained from both temperature-jump and stopped-flow experiments are shown in Figure 3. In almost all of the systems

Table I: Initial Concentrations and Experimental and Calculated Relaxation Times[a]

no.	NPI[e] × 10^4 (M)	BPB[f] × 10^5 (M)	pH[b]	τ_1 (ms)[c]		τ_2 (ms)[c]	
1	2.95	1.00	3.78	9.6[s]	(10.7)		(20.0)
2	2.95	0.50	3.80	10.0[s]	(11.0)		(19.6)
3	2.50	1.00	3.78	11.9[s]	(11.6)		(21.7)
4	1.91	1.00	3.86		(13.7)	21.3[s]	(25.0)
5	1.70[d]	1.00	3.77		(13.7)		(26.3)
6	1.40	1.00	3.87	16.1[s]	(15.9)		(29.4)
7	1.40	0.50	3.86	14.3[s]	(15.9)		(27.8)
8	1.08	1.00	3.78		(16.7)	26.3[s]	(30.3)
9	1.05	1.00	3.89		(17.9)	29.4[s]	(30.3)
10	1.00	1.00	3.78	17.2	(17.2)		(34.5)
11	0.95	2.00	3.77		(16.7)	31.2	(30.0)
12	0.85[d]	1.00	3.78		(18.2)	34.5	(30.0)
13	0.85[d]	0.50	3.80		(18.9)	34.5	(35.7)
14	0.75	1.00	3.84		(19.6)	34.5[s]	(38.5)
15	0.71	0.30	3.20	18.2[s]	(16.4)		(30.0)
16	0.71	0.30	3.83		(20.8)	35.7[s]	(30.0)
17	0.54	1.00	3.77		(21.3)	40.0[s]	(45.5)
18	0.53	1.00	3.84		(22.2)	30.0[s]	(45.5)
19	0.50	1.00	3.80		(22.2)	47.6	(47.6)
20	0.42[d]	1.00	3.80		(23.3)	50	(50.0)
21	0.30	1.00	3.94		(27.0)	71.4[s]	(55.6)
22	0.24	4.00	3.82		(20.4)	76.9	(71.4)
23	0.24	2.00	3.83		(25.0)	62.5	(66.7)
24	2.30	1.00	2.73	6.9	(8.5)	22.7	(22.2)
25	2.30	1.00	3.16	7.8	(9.1)	23.3	(22.2)
26	2.30	1.00	3.74	14.3	(11.8)		(22.7)
27	2.30	1.00	4.01	14.9	(14.1)		(22.7)
28	0.60	0.50	2.77	17.5	(16.7)		(40.0)
29	0.60	0.50	3.12	20.0	(17.2)		(40.0)
30	0.60	0.50	3.79		(21.7)	38.5	(40.0)
31	0.60	0.50	4.00		(23.8)	38.5	(41.7)

[a] Results from stopped-flow experiments are indicated by a superscript "s". [b] [H] was calculated by dividing the measured hydrogen ion activity by γ^H (~0.8). [c] Experimental values are given first. Values in parentheses were calculated via eq 2 by using k_{1f} and k_{2f} as given in the text. [d] Nitrated neurophysin I, prepared according to Breslow & Gargiulo (1977). [e] NPI, neurophysin I. [f] BPB, bromophenol blue.

examined, a single relaxation time could be extracted from the data (Table I). In one system at pH 2.73 and 3.16, it was possible to decipher two coupled relaxation times (Table I, no. 24 and 25). As the concentration of neurophysin I was decreased, both relaxation times became slower (Table I, no. 1–23). Changes in bromophenol blue concentration had little or no effect upon the observed relaxation time. Nitration of the tyrosine of neurophysin I had no effect on the observed relaxation time (Table I, no. 5, 12, 13, and 20). As the pH was increased from pH 2.7 to 4.0, the relaxation time increased (Table I, no. 24–27 and 28–31).

In order to formulate a mechanism for these data, I had to consider the following possible interactions: neurophysin dimerization, neurophysin–bromophenol blue interactions, and proton-transfer reactions of both neurophysin and bromophenol blue. The dependence of the relaxation time on neurophysin concentration indicated that this species must be intimately involved in any proposed mechanism. The small influence of bromophenol blue concentration on the relaxation time was somewhat puzzling in view of the observation of its ability to bind neurophysin (Figure 1 and Figure 2). However, a coupling of neurophysin dimerization with binding to the dye could result in a relaxation spectrum which was more strongly dependent on the concentration of protein than of dye. No explicit information is available on the pH dependence of neurophysin dimerization. However, there exists indirect evidence that suggests the dimerization is probably not pH dependent. A comparison of neurophysin sedimentation

Scheme I

$$\begin{array}{ccc} \mathrm{NP} + \mathrm{NP} \underset{k_{1r}}{\overset{k_{1f}}{\rightleftharpoons}} (\mathrm{NP})_2 & & \\ + & & \\ \mathrm{HIn}^- \overset{K_{IN}}{\rightleftharpoons} \mathrm{H}^+ + \mathrm{In}^{2-} & & (\mathrm{A}) \\ k_{2r} \uparrow\downarrow k_{2f} & & \\ \mathrm{NPH}^+ \cdots \mathrm{In}^{2-} & & \end{array}$$

velocity at pH 6 and 2 does not indicate an increase in dimerization at low pH (Breslow & Gargiulo, 1977). Kinetic studies at pH 7.4 were consistent with the value of the dimerization constant determined at pH 5.8 (Pearlmutter & McMains, 1977). The binding of neurophysin I to bromophenol blue at pH 3.8 can be expressed as a linear Scatchard plot when utilizing the dimerization constant determined at pH 5.8 (Figure 2). Were neurophysin dimerization pH dependent, it might be possible to monitor the dimerization with a pH indicator other than bromophenol blue, unless the relaxation amplitude resulting from pH coupling alone were small. As previously mentioned, no relaxation effect could be observed with a mixture of neurophysin I and methyl orange, which has a pK within 0.1 of bromophenol blue. For these reasons, I think it to be unlikely that the pH dependence of the relaxation time is a reflection of a pH dependent dimerization.

Proton-transfer reactions between bromophenol blue and pyridine bases have been observed to occur in the millisecond time region (Crooks & Robinson, 1970, 1971). These reactions were shown to occur between HIn^- and the base to form an ion pair where the charges are redistributed; this may be shown schematically as $HIn^- + B = In^{2-} \cdots HB^+$ (Crooks & Robinson, 1970). Thus, although the base, in this system neurophysin I, is reacting with the protonated bromophenol blue, the ion-pair product could have spectral characteristics of the unprotonated dye. This is, in fact, the case for the bromophenol blue–neurophysin I interaction (Figure 1).

The mechanism which accounts for the relaxation data and is consistent with the known properties of all species in the system involves the dimerization of neurophysin coupled to the interaction of neurophysin I with the protonated bromophenol blue to form an ion-pair complex. This is shown in Scheme I, where NP represents neurophysin I monomer, $(NP)_2$ represents the dimer, HIn^- and In^{2-} represent the protonated and unprotonated forms of bromophenol blue, respectively, and $NPH^+\cdots In^{2-}$ represents the ion-pair complex formed between the neurophysin monomer and the HIn^- form of the dye. Fast proton transfers are indicated by equal signs, and the reactions being monitored are shown by the arrows. The two relaxation times for mechanism A are obtained by casting the rate equations for restoration of equilibrium in the form of a determinant (Eigen & DeMaeyer, 1963) whose roots may be expressed as

$$\frac{1}{\tau_{1,2}} = \frac{1}{2}[(a_{11} + a_{22}) \pm \sqrt{(a_{11} + a_{22})^2 - 4(a_{11}a_{22} - a_{12}a_{21})}] \quad (2)$$

where τ_1 corresponds to the (+) sign and τ_2 corresponds to the (−) sign. The a_{ij} factors, which are calculated from preequilibrium and mass-balance considerations, are $a_{11} = 4k_{1f}[\overline{NP}] + k_{1r}$, $a_{12} = 2k_{1f}[\overline{NP}]$, $a_{21} = k_{2f}[\overline{HIn^-}]$, and $a_{22} = k_{2f}[\overline{NP}/(1 + \alpha) + \overline{HIn^-}] + k_{2r}$ where $\alpha = K_{IN}/(\overline{H} + \overline{In^{2-}})$. The stability constants used to calculate the equilibrium concentrations are $K_{IN} = 1.41 \times 10^{-4}$ M (Yapel & Lumry,

Scheme II

$$\begin{array}{ccc} NP + NP \underset{k_{1r}}{\overset{k_{1f}}{\rightleftharpoons}} (NP)_2 & & \\ + & & \\ HIn^- & \overset{K_{IN}}{\rightleftharpoons} & H^+ + In^{2-} \\ K_B \Big\| & & \\ NPH^+ \cdots In^{2-} & & \end{array} \qquad (B)$$

1971), $K_B = 5.36 \times 10^4$ M^{-1}, and $K_D = 7.7 \times 10^3$ M^{-1} (Nicolas et al., 1976). The bars indicate equilibrium concentrations.

The rate constants which gave the best fit to the experimental data were determined in the following manner. Estimates for the best trial values of k_{1f} and k_{1r} were obtained by plotting $1/\tau$ (experimental) vs. the concentration function which would be obtained if bromophenol blue binding were fast compared with dimerization. This is a reasonable approximation because the lack of concentration dependence of the relaxation time on bromophenol blue indicates that this species is probably not involved in the most significant rate-determining reaction. This was confirmed in the complete analysis which is described later. The mechanism is shown in Scheme II where K_B is the binding constant for neurophysin–dye binding, 5.36×10^4 M^{-1}, and K_{IN} is the indicator dissociation constant, 1.41×10^{-4} M. The relaxation expression for mechanism B is

$$\frac{1}{\tau} = k_{1f}\left[4\overline{NP}\frac{1 + K_B[\overline{NP}/(1+\alpha)]}{1 + K_B[\overline{NP}/(1+\alpha) + \overline{HIn}]}\right] + k_{1r} \qquad (3)$$

where the symbols have the same meaning as before. When the data were plotted by using this equation, a straight line was obtained only for $1/\tau$ values less than 50 s^{-1}. This allowed the calculation of the forward and reverse rate constants for the neurophysin dimerization, 9.2×10^4 M^{-1} s^{-1} and 12 s^{-1}, respectively. These values were then used in eq 2 to solve the complete relaxation expression.

The data of Table I were fitted to mechanism A by inserting a number of combinations for the unknown rate constants, k_{2f} and k_{2r}, into eq 2 by means of a computer program and then comparing the calculated values for τ_1 and τ_2 with those observed experimentally. The best fit was determined by minimizing the quantity σ, the error function, defined by

$$\sigma = \frac{1}{N}\sum\left|\frac{\tau^{-1}_{\text{exptl}} - \tau^{-1}_{\text{calcd}}}{\tau^{-1}_{\text{exptl}}}\right|$$

where N is the number of data points, exptl is the experimental value, and calcd is the calculated value. The best fit rate constants were found to be $k_{1f} = 9.2 \times 10^4$ M^{-1} s^{-1}, $k_{1r} = 12$ s^{-1}, $k_{2f} = 1.1 \times 10^6$ M^{-1} s^{-1}, and $k_{2r} = 20$ s^{-1}. A comparison of experimental and calculated values for τ_1 and τ_2 is shown in Table I.

The dye binding to neurophysin I has a forward rate constant 12 times greater than the forward rate constant for neurophysin dimerization. This confirms the initial approximation technique which was based on the assumption that dimerization is slower than dye binding.

If the only reaction under observation was the binding of bromophenol blue to neurophysin I, i.e., NP + HIn$^-$ = NPH$^+\cdots$In^{2-}, then one would expect the relaxation time to fit the relationship $\tau^{-1} = k_f(\overline{NP} + \overline{HIn^-}) + k_r$. However, with this mechanism one obtains a large deviation from linearity when plotting $1/\tau$ vs. $\overline{NP} + \overline{HIn^-}$. Also, this simple mechanism cannot be reconciled with the fact that mixing neurophysin I with bromophenol blue in the stopped-flow spectrophotometer results in relaxation times which are independent of dye concentration (Table I, no. 1 and 2 and 6 and 7).

Table II: Rate Constants for Bovine Neurophysin I Binding Systems at $I = 0.1$ and 25 °C

reaction	k_f (M^{-1} s^{-1})	k_r (s^{-1})	ref
NP + HOXY[c]	0.8×10^5	2	*a*
NP + HLVP[c]	3.6×10^5	7	*a*
$(NP)_2$ + HOXY	28.0×10^5	11	*a*
$(NP)_2$ + HLVP	23.0×10^5	15	*a*
NP + NP	0.9×10^5	12	*b*
NP + HIn$^-$	11.0×10^5	20	*b*

[a] Pearlmutter & McMains (1977). [b] This paper. [c] HOXY is protonated oxytocin; HLVP is protonated lysine-vasopressin.

It is possible to fit the data to a mechanism which involves a fast binding of bromophenol blue to the neurophysin I which is followed by a slower, rate-determining conformational change. The initial complex formed would be an intermediate hydrogen-bonded complex, analogous to that formed with bromophenol blue and pyridine bases (Crooks & Robinson, 1970, 1971), and the rate-determining step would involve a conformational change of the complex to form the final ion-pair product.

This mechanism fails to account for the pH dependence of the relaxation time. If I ignore the pH dependence and try to fit only the data obtained at pH 3.8, a binding constant for neurophysin–dye binding of from 10^7 to 10^8 M^{-1} is required; this is not possible from the equilibrium binding studies shown in Figure 2. Therefore, I have eliminated this mechanism as a realistic possibility.

Discussion

The changes in spectrum which occur upon mixing bromophenol blue with bovine neurophysin I follow a pattern similar to that seen for the interaction of α-chymotrypsin and β-lactoglobulin at pH 3.7 with the dye (Koren & Hammes, 1976). In all of these systems, mixing of the dye with the protein results in a decrease in absorption of the acid form and an increase in absorption of the basic form of bromophenol blue. In the neurophysin I system, it was necessary to include dye binding as part of the detailed mechanism because these two reactions were kinetically coupled. This was not the case with the α-chymotrypsin and β-lactoglobulin systems (Koren & Hammes, 1976).

A summary of the rate constants which have been determined for some bovine neurophysin I systems is shown in Table II. Bromophenol blue binding to the monomer form of neurophysin I occurs 14 times faster than oxytocin binding and 3 times faster than lysine vasopressin binding; this may be a reflection of the formation of an ion-pair hydrogen-bonded complex where the redistribution of charges contributes to the effective collision rate. Because both native and mononitrated neurophysin I exhibit identical kinetic profiles upon binding to bromophenol blue (Table I), I can eliminate any significant participation of the neurophysin tyrosine residue on the binding reaction. The lack of reactivity of the neurophysin I dimer with bromophenol blue may occur because the residues on the protein which bind the dye become less accessible upon dimerization. Because the three-dimensional structure as well as the location of the disulfide bonds of neurophysin I are unknown, I can only speculate on the nature of the amino acid residues involved in dye binding. Since neurophysin I contains a terminal α-carboxy residue, which is presumably not protonated at pH 3.8 (Breslow et al., 1971), it is possible that the

Table III: Second-Order Rate Constants for Protein Dimerization Interactions

system	k_f (M^{-1} s^{-1})	ref
insulin	1.1×10^8	*a*
α-lactoglobulin	3.7×10^4	*a*
β-chymotrypsin	3.7×10^3	*a*
deoxyhemoglobin dimer	6.3×10^5	*b*
phosphofructokinase	8.0×10^3	*c*
neurophysin I	9.2×10^4	*d*

[a] Koren & Hammes, 1976. [b] Anderson et al., 1971. [c] Parr & Hammes, 1976. [d] This paper.

initial interaction occurs at this residue. After initial formation of the complex, the ion pair may be stabilized by the hydrogen bonding of the phenolic hydrogen to one of the many negatively charged glutamic acid residues known to be present on neurophysin (Wuu & Crumm, 1976).

Neurophysin dimerization does not involve tyrosine-49, since both native and mononitrated neurophysin are identical in both the equilibrium binding and kinetic studies. The forward rate constant for neurophysin I dimerization is typical of those for many other protein systems where k_f values range from 3.7×10^3 to 6.3×10^5 M^{-1} s^{-1} (Table III). Insulin, which has a k_f of 1.1×10^8 M^{-1} s^{-1} (Koren & Hammes, 1976), is close to diffusion-controlled, but this protein appears to be an exception. Neurophysin I dimerization, like most other self-associating protein systems, is not diffusion-controlled. The lack of a pH dependence for neurophysin I dimerization strongly indicates that this interaction is stabilized by hydrophobic interactions between the monomer units.

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