Interactions of Oxytocin and Vasopressin with Bovine Neurophysins I and II

EFFECTS OF HORMONE BINDING ON THE PROTEIN QUATERNARY STRUCTURE: A SIMPLE MODEL*

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Pierre Nicolas, Maryse Camier, Philippe Des sen,‡ and Paul Cohen§

From the Groupe de Neurobiochimie Cellulaire et Moléculaire, Université Pierre et Marie Curie, 75006 Paris; Service de Biochimie du Département de Biologie, Centre d’Études Nucléaires, SACLAY, Gif-sur-Yvette; and §Laboratoire de Biochimie, École Polytechnique, 91120 Palaiseau, France

The effects of hormone binding on the reversible monomer ↔ dimer equilibrium of bovine neurophysins I or II in solution have been studied by sedimentation equilibrium measurements performed in conjunction with equilibrium dialysis experiments. Under normal solution conditions saturating amounts of oxytocin displace the neurophysin dimerization equilibrium toward the associated form of the protein to give a dimeric complex with two oxytocin molecules bound per dimer. Vasopressin exerts different influences on this oligomerization process. At low fractional saturation this ligand exhibits a behavior similar to oxytocin with a higher affinity for the neurophysin dimer than the monomer. But in contrast, at higher fractional saturation, vasopressin strongly displaces the aggregation equilibrium toward a monomeric complex bearing two vasopressin molecules. However, in the presence of a high concentration of LiCl two oxytocin molecules are bound per neurophysin protomer (10,000 daltons). These observations, together with earlier data for vasopressin binding, suggest that each neurophysin molecule possesses two structurally distinct hormone binding sites. These observations can be rationalized in a simple schematic model of hormone binding to neurophysin in which oxytocin favors a dimeric form with one hormone binding site available per 10,000 daltons while vasopressin favors the monomeric form with two hormone binding sites available per 10,000 daltons.

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‡ To whom correspondence should be addressed at Groupe de Neurobiochimie Cellulaire et Moléculaire, Université Pierre et Marie Curie, 96 Boulevard Raspail, 76006 Paris, France.

§ Contribution of cooperativity to the binding process; and (d) the existence of differences between oxytocin and vasopressin in the mode of complex formation (2, 12). Although most authors seemed to agree that, at equilibrium, only one oxytocin molecule could be bound per 10,000 daltons (1, 3), work in this laboratory had first shown that two vasopressin molecules could be bound per 10,000 daltons with apparent similar affinities (1, 2). This unusual difference between the behavior of oxytocin and vasopressin binding has not yet been clearly explained. In order to clarify this problem we have further investigated several aspects of the binding process using equilibrium dialysis under various conditions in connection with corresponding rigorous measurements of the apparent weight average molecular weight exhibited by the protein-hormone complexes. The observations we report here strongly suggest that each neurophysin molecule of 10,000 daltons possesses two distinct hormone binding sites which can be occupied by oxytocin or vasopressin under certain conditions. We show that the relative capacity of oxytocin, or vasopressin, to bind at the second hormone binding site is correlated with specific differential effects of hormone binding on the neuro-
Physin quaternary structure, i.e. the monomer = dimer equilibrium exhibited by the protein in solution at pH 5.60. These results, and previous observations (1, 2), can now be rationalized in a simple schematic model for hormone binding to neurophysin.

**MATERIALS AND METHODS**

**Neurophysins**—Highly purified bovine neurophysins I and II were prepared as previously described (1) by isoelectric focusing from an acetone powder of freshly collected bovine pituitaries. The samples routinely tested for light content were found to contain no more than 0.5% by weight of glyceride derivatives as judged by gas chromatographic analysis of the fatty acid methyl esters produced after alkaline hydrolysis of the samples (16). Neurophysin samples were routinely tested for homogeneity using gel electrophoresis, gel isoelectric focusing, and amino acid composition.

**Hormone**—Oxytocin and vasopressin were generous gifts of Sandoz (Basle). The tritiated hormones (10 to 30 Ci/mmol) were also prepared as previously reported (17, 18) and routinely tested for their pharmacological activities and their radiochemical purity by the usual electrophoretic and chromatographic tests (17, 18).

**Equilibrium Dialysis**—Hormone binding studies were run at 24°, pH 5.60 (0.1 M sodium acetate buffer), as described in detail previously (1). Refined analysis of the Scatchard plots required that a minimum of 9 or 10 different hormone concentrations be tested (from 5 x 10^{-4} M to 10^{-3} M) at each protein concentration. Each point was run in triplicate. Counting of the radioactive samples was done by means of a liquid scintillation spectrometer (Intertechnique SL 30).

Concentrations of neurophysins were evaluated on a Cary 118 C spectrophotometer assuming an ε_{260} = 3400 cm^{-1} M^{-1} at 260 nm. Fractional saturation ratios s were expressed as bound ligand (C), concentrations per protein molar concentration considering the molecular weights of neurophysins I and II equal, respectively, to 9,560 and 10,041 (19, 20). C, is the free ligand concentration.

**Sedimentation Equilibrium Experiments**—The measurements were made using a Beckman Spinco model E analytical ultracentrifuge and an equilibrium cell with two compartments. Protein, or protein plus hormone, samples were put in one compartment while the buffer alone, or the hormone in the buffer, was in the other compartment. Neurophysin concentration was 0.4 or 0.8 mg/ml in the absence of ligand, and 0.6 mg/ml in the presence of peptide. Ultraacentrifugation was performed at 20° at 30,000 rpm for 6 days. After 96 hours, the solution distribution was checked to see whether equilibrium was reached. The protein distribution was obtained using Rayleigh interference optics. The interferometric optics were aligned using the method of Richarz and Schachman (21).

**Circular Dichroism Spectra**—Circular dichroism spectra were recorded in a Jasco dichrograph III, with a sensitivity of 10^{-4} A units/mm, using 0.1-cm optical pathway cuvettes and a protein concentration of 0.5 mg/ml.

**RESULTS**

**Sedimentation Equilibrium Measurements of Molecular Weight of Neurophysin II in Presence and Absence of Added Oxytocin**—At pH 5.60, in the absence of added ligand, the plot of In concentration of neurophysin II versus ΔR^2 (Fig. 1) gives a curve typical of a self-associating system. The inset represents the observed values of the weight average molecular weight (Mw) at each concentration. The solid line represents the theoretical variation of Mw with concentration for a monomer = dimer equilibrium with an association constant X, = 5.8 x 10^3 M^{-1}. These values are in very good agreement with that previously reported by Brestory et al. (15) for neurophysin II at pH 8.20. They indicate that the association is relatively weak between the protomers of the dimeric structure. The association constant X, was verified by measurements carried out at two different protein concentrations (0.4 mg/ml and 0.8 mg/ml, respectively).

At pH 5.60, when saturating amounts (1 mg/ml) of oxytocin were added to the neurophysin II solution at concentrations approximating 0.5 mg/ml, i.e. where the protein alone exhibits a Mw approaching 12,500 (Fig. 1) the observed plots of In concentration of neurophysin II versus ΔR^2 indicated unambiguously the presence of a single molecular species in solution with an apparent Mw of 21,500 ± 1075 over a range of concentrations from 0.05 to 1.5 mg/ml. This result demonstrated that oxytocin displaces strongly the monomer = dimer equilibrium of neurophysin II toward the higher molecular weight species. Thermodinamically this situation indicates that the affinity of oxytocin for the associated dimer form of neurophysin II is higher than for the monomeric protein.

**Binding of Oxytocin to Neurophysin II at Various Protein Concentrations**

![Graph showing binding of oxytocin to neurophysin II](http://www.jbc.org/)

**FIG. 1.** High speed sedimentation equilibrium of native bovine neurophysin II in 0.1 M sodium acetate buffer, pH 5.60, at 20°: O, in the absence of oxytocin (initial concentration of protein 0.8 mg/ml); ●, in the presence of a saturating amounts (10^{-3} M) of oxytocin (initial concentration of protein 0.5 mg/ml). As the latter experiment was done at 30,000 rpm instead of 50,000 rpm for the former, the points were replotted for 30,000 rpm with arbitrary position along the ΔR^2 axis. Inset, replots of the curve (O) in terms of weight average molecular weight (Mw) versus concentration. The solid line represents the theoretical variation of Mw versus concentration calculated assuming an apparent monomer = dimer equilibrium with an association constant X, = 5.8 x 10^3 M^{-1}.
Concentrations—When the binding of oxytocin (from $5 \times 10^{-7}$ M to $10^{-3}$ M) to neurophysin II at various concentrations ranging from 5 $\mu$M to 0.3 mM was measured and the data plotted according to Scatchard (24), typical curves were obtained (Fig. 2). The type of curvilinearity observed is characteristic of ligand binding-coupled effects on a self-polymerizing protein (25-28). In all cases the maximal observed values of the fractional saturation were close to 1.0. Since the previously measured effects of oxytocin on the $M_w$ of the protein indicates that, at saturation, the neurophysin-oxytocin complex is dimeric, this proves that the dimeric form of neurophysin II under these conditions possesses two hormone binding sites occupied by oxytocin. In addition, the observation, from the sigmoidal plots of Fig. 2, that the average slope of the curves increases with increasing protein concentrations strongly confirms the previous evidence that the affinity of oxytocin for the neurophysin dimer is higher than for the monomeric form of the protein.

Binding of (Lys*) Vasopressin to Neurophysin II—Refined analysis of the data for vasopressin binding to neurophysin II indicated the existence of curvature in the lower and upper parts of the Scatchard plots (Fig. 3). As in the case of oxytocin, some positive cooperativity could be observed at low $\tilde{v}$ values ($\tilde{v} < 1$), and the upper part of these plots is essentially similar to those obtained for oxytocin. In the second part of the binding isotherms, there is observed for high $\tilde{v}$ values ($\tilde{v} > 1$), a reverse effect indicative that neurophysin II could be saturated by the binding of a second hormone molecule. This result was repeatedly observed with different biologically active preparations of tritiated vasopressin. However, in our hands, it was found that the capacity of binding a second vasopressin molecule was lessened or eventually was no longer detected when samples of tritiated vasopressin with a decreased biological activity were tested. In that case, a behavior very much like that of oxytocin was observed, indicative that structural changes occurring in these hormone samples led to a loss of the vasopressin character of the molecules. Clearly these observations confirm previous results that using fully biologically active vasopressin under carefully controlled conditions, neurophysin II can bind two vasopressin molecules per protein monomer of 10,000 daltons (1). The affinity of the second peptide ligand molecule appears to be lower than that of the first molecule. Taken together those features for vasopressin binding to neurophysin II and the previous observations made with oxytocin strongly suggest that ligand binding may have different effects on the proportion of either the associated, or dissociated, neurophysin species in solution at different $\tilde{v}$ ratios.

Equilibrium Sedimentation Studies of Neurophysin II in Presence of (Lys*) Vasopressin—To test for this possibility, the weight average molecular weight ($M_w$) of neurophysin II was measured in the presence of vasopressin concentrations corresponding respectively to $\tilde{v} = 0.9$ and $\tilde{v} = 1.5$ on the Scatchard plots (neurophysin II concentration was 50 $\mu$M) (Fig. 3). For such an experiment, vasopressin (10 $^{-3}$ M) was added to the neurophysin II solution (initial concentration 0.5 mg/ml) at pH 5.60. Fig. 4 clearly shows that, under these conditions, the predominant molecular species is dimeric. For instance at a protein concentration of 0.5 mg/ml, i.e. the conditions of the equilibrium dialysis experiments, the observed value of $M_w$ is 22,000 $\pm$ 1100. These results again demonstrate the dramatic effects of ligand binding on the $M_w$ of the protein. In contrast, when the same initial concentration of neurophysin II was exposed to a 10-fold excess of (Lys*) vasopressin (10 $^{-3}$ M), the curve obtained indicated clearly that the proportion of dimeric complexes was significantly lower (Fig. 4). Up to a protein concentration of 0.5 mg/ml the complex was essentially monomeric. Thus, at a protein concentration of 0.5 mg/ml, the conditions of the equilibrium dialysis where the protein alone is expected to have a $M_w = 12,500$, the $M_w$ of the complex was 14,300 $\pm$ 715. But in the lower part of the liquid column, in the centrifugation cell, the proportion of the higher molecular weight species increased, since the vasopressin:neurophysin ratio decreased. In conclusion, vasopressin behaves very much like a monomer under these conditions, and this is confirmed by the $M_w$ measurements, where the ratio 1:1 x $10^{3}$ M$^{-1}$

![Fig. 2. Scatchard plots of the binding of oxytocin (from 5 x 10^{-7} M to 10^{-3} M) in 0.1 M sodium acetate buffer, pH 5.60, at 24 $\pm$ 1$^\circ$, to various concentrations of neurophysin II: A, neurophysin II (5 x 10^{-7} M); O, neurophysin II (6 x 10^{-7} M); solid lines are theoretical relationships obtained by a least squares procedure based on Equation 7x (see "Appendix") with $X_o = 5.8 \times 10^4$ M^{-1}.](image1)

![Fig. 3. Scatchard plots of the binding of (Lys*) vasopressin (from 5 x 10^{-7} M to 10^{-3} M) to neurophysins I and II in 0.1 M sodium acetate buffer, pH 5.60, at 24 $\pm$ 1$^\circ$. O, neurophysin II (5 x 10^{-7} M); C, neurophysin I (5 x 10^{-7} M). Inset, variation of the weight average molecular weight ($M_w$) of the bovine neurophysin I versus concentration: in 0.1 M sodium acetate buffer, pH 5.60, at 20$^\circ$. The solid line represents the theoretical variation of $M_w$ versus concentration calculated assuming an apparent monomer = dimer equilibrium with an association constant of $7.7 \times 10^4$ M$^{-1}$.](image2)
like oxytocin for low vasopressin:neurophysin ratios, i.e. exhibits a higher affinity for the dimer. But the opposite effect was detected as the proportion of vasopressin increased, and the complex was found to be predominantly monomeric in solution.

### Sedimentation Equilibrium Measurements of Molecular Weight of Neurophysin I

At pH 5.60, the plot of ln concentration of neurophysin I versus $\Delta R^2$ gives also a curve typical of a self-associating system and the inset of Fig. 3 represents the observed values of the weight average molecular weight ($\bar{M}_w$) at each concentration. The solid line represents the theoretical variation of $\bar{M}_w$ with concentration for a monomer = dimer equilibrium with an association constant of $7.7 \times 10^6$ M$^{-1}$. The behavior of both neurophysins I and II was essentially similar except that the $\bar{M}_w$ increased slightly faster with increasing protein concentration in the case of neurophysin I compared with neurophysin II, indicative of a higher monomer-monomer affinity in the first case.

### Binding of (Lys) Vasopressin to Neurophysin I

In this case, the Scatchard plots were slightly different from those of neurophysin II (Fig. 3). The curvature was less marked and only a small difference in the slope of the binding isotherm was observable at high versus low $v$ ratio. This suggests that the ligand has probably very similar effects on the oligomerization of neurophysin II and neurophysin I, but that for the latter, vasopressin may have less pronounced effects on the capacity of the protein to bind a second hormone molecule.

### Binding of Oxytocin to Neurophysin II in Presence of LiCl

When the binding of oxytocin (from $10^{-4}$ M to $10^{-2}$ M) to neurophysin II (50 µM) at pH 5.60 was measured in the presence of 1.4 M LiCl, the Scatchard plot obtained (Fig. 5) shows unambiguously that two oxytocin molecules could be bound, at saturation, per protein fraction of 10,000 daltons and that the affinity of the ligand for the neurophysin II was now significantly lower than for the protein in absence of LiCl. Preliminary experiments showed that, for oxytocin binding to neurophysin II, the maximum fractional saturation ratio was obtained for LiCl concentrations close to 1.40 M. Above this concentration LiCl had an opposite effect and the $\bar{M}_w$ apparently decreased from 2 to 1. CD measurements between 235 nm and 360 nm indicated that up to LiCl concentrations of 2.5 M no major changes in the spectrum appeared. Above this LiCl concentration a significant decrease of the magnitude of the 248 nm and 278 nm ellipticities appears and suggests a collapse in the tertiary structure of the protein. Since LiCl seemed to favor the unmasking of oxytocin sites we tested the hypothesis that this effect might be brought about by means of an action of the salt on the oligomerizing protein. Sedimentation equilibrium measurements run in 1.40 M LiCl in the absence or in the presence of saturating amounts of oxytocin showed that this salt had no measurable effects on the reversible monomer = dimer equilibrium of the protein above or on the dimeric state of the hormone-neurophysin complex. However, the partial specific volume of the neurophysin II shifted from 0.709 to 0.662 in 1.40 M LiCl.

### DISCUSSION

It is now firmly established that bovine neurophysins I and II exist in a thermodynamically reversible monomer = dimer equilibrium in solution at pH 5.60, i.e. the pH at which the binding studies were run. This provides a simple explanation of the fact that previous tentative evaluations of the molecular weight of the proteins by sedimentation velocity measurements led to different values depending upon the neurophysin concentration at which those experiments were performed. Hope et al. (29) recently reported on the dimeric nature both of the hormone-neurophysin II complex and of the protein alone from simple measurements of sedimentation velocities. These authors claimed that ligand binding induced only conformational rearrangements of the pre-existing dimeric protein. This is in contrast with the previous report of Beslow et al. (15) of a monomer = dimer equilibrium and with the above reported evidence that ligand drastically affects this equilibrium. One possible explanation for these discrepancies may reside in the fact that these authors worked with an initial protein concentration sufficiently high to have a large proportion of dimer in solution.

It is clear that even in binding measurement conducted at very low protein concentration (0.5 or 0.05 mg/ml) where the proportion of dimer in the absence of ligand is relatively small (Fig. 1), some sign of positive cooperativity could be detected in the binding isotherms. These phenomena in multiaubunit protein systems are known to be due to modulation of the strength of subunit interactions coupled to ligand binding (27-30). In some cases, ligand binding promotes association of...
the subunits, but in others, the opposite effects can be detected. The above reported observations demonstrate that oxytocin strongly displaces the monomer = dimer equilibrium of the protein in favor of the associated form. Thermodynamically this effect is brought about by the fact that the hormonal ligand has a higher affinity for the dimer than for the monomer. This conclusion is substantiated by the observation that the apparent binding constant is higher, the higher the neurophysin concentration, consequently the proportion of dimer. This gives a satisfactory explanation to the reported discrepancies between the $K_2$ values previously measured in our laboratory (1) at low neurophysin concentrations (0.5 mg/ml) compared with those of other authors (3, 4) carried out at protein concentrations close to 2 or 6 mg/ml. Since a maximum of one molecule of oxytocin could be bound per protein fraction of $M_r = 10,000$ at saturation, and considering the fact that, for this value of $\tilde{\tau}$ the protein is predominantly in the associated, dimeric form it is clear that the neurophysin dimer possesses two occupied hormone sites at saturation.

The situation in the case of (Lys$^8$) vasopressin binding is rather more complicated since opposite effects on the protein quaternary structure could be observed over the saturation process. At low values of $\tilde{\tau}$ clearly the behavior of (Lys$^8$) vasopressin is very much like oxytocin and the hormone exhibits a preference for binding to the dimeric neurophysin. Consequently a large proportion of dimeric complex ($MW = 22,000$) was found in the presence of vasopressin and when values of $\tilde{\tau}$ were smaller than 1.0. In contrast, at a ligand concentration closer to the 2:1 hormone to protein stoichiometry, a reverse process was induced by ligand binding. In that case the vasopressin-neurophysin complex was obviously monomeric. It is now possible to describe the noncovalent association of oxytocin and (Lys$^8$) vasopressin to bovine neurophysin II at pH 5.69 as a system where ligand binding is coupled to the association of two monomers with four available binding sites into a dimer possessing two available sites. The scheme of Fig. 6 represents this phenomenon tentatively. $K_1$, $K_2$, and $K_3$ are, respectively, the association constants for the successive equilibria, and $X_3$ is the equilibrium constant for the dimerization reaction. The essential features of this schematic representation are the following: (a) the existence of individual monomeric protein molecules possessing two structurally distinct and, possibly, thermodynamically nonequivalent sites; (b) the possibility that these monomers associate noncovalently as a dimer made of two equivalent protomers and possessing only two sites, these two sites exhibiting a higher affinity for oxytocin than the original sites of the nonassociated protomer; (c) it is presumed that two out of the four sites of the dimer, in some fashion and possibly consecutive to conformational rearrangements associated with dimerization, are made unavailable to ligand binding.

This is a very simple representation of hormone association to the oligomerizing protein. This model was tested and values of the association constants $K_1$ and $K_3$ were evaluated in the case where the ligand is oxytocin (see "Appendix"). They were, respectively, found to be equal to $K_1 = 5 \times 10^4 \text{M}^{-1}$, $K_3 = 2.5 \times 10^4 \text{M}^{-1}$. However, for very low $\tilde{\tau}$ values attempts to get the best curve fit with the model lead to rather poor results, suggesting that the cooperativity associated with dimerization cannot account for the curvature observed at $\tilde{\tau} < 0.25$. In the case where the ligand is vasopressin ($L'$), $K_1$ and $K_3$ were assumed to be equal to the corresponding values obtained with oxytocin. Given this approximation, a value of $K'_1$ was obtained and found to be equal to $0.8 \times 10^3 \text{M}^{-1}$.

Although we have no experimental evidence that neurophysin I quaternary structure is similarly affected by ligand binding, it seems reasonable to assume that most of the steps involved in the present model are also applicable to neurophysin I. In this scheme, it can be seen that only the $DL'$ form can be attained in solution in the case of oxytocin. It was not possible to assess the existence of a monomeric neurophysin bearing two oxytocin molecules. However, results obtained with LiCl suggest that the binding of two more oxytocin molecules may occur on a rearranged dimer. In the case of (Lys$^8$) vasopressin the monomeric form $ML'$ was shown to exist in solution at high ligand binding ratio. This can be rationalized, referring to the scheme proposed in Fig. 6, assuming that the $DL'$ dimeric complex is less stable than the $DL'$ complex. Alternatively, this might simply mean that the vasopressin molecules exhibit a higher affinity for the $ML'$ form (which gives $ML''$) than for the $DL'$ complex. Thermodynamically these two alternatives are rigorously equivalent.

Many questions arise relative to the striking difference exhibited by the two hormones and the biological significance of such a behavior. One possible explanation is that, in the case where vasopressin is the ligand, the instability of the $DL'$ complex is brought about by the repulsive charges introduced by the ε amino group of amino acid residue 8. The use of adequate nonapeptide analogs may allow more accurate description of this feature.

With respect to the number of binding sites, the discussion of the internal duplication of amino acid sequences (31) originally made by Camier et al. (1) to explain the observed multiplicity of vasopressin binding sites becomes relevant. The presence of two hormone binding sites per 10,000 daltons makes the possibility that tyrosine 49 may be part of the binding site rather unlikely. It reinforces the simple idea that perturbations of this chromophore accompanying binding may only reflect the conformational changes associated with this residue (2, 32).

Consistent with the finding of a second oxytocin binding site in the presence of LiCl is the recent observation (33) that a second binding site for the tripeptide analog of the $\text{NH}_2$
The terminus of the hormone can be revealed after neurophysin has been covalently attached to poly-D,L-Ala-L-Lys. Although the mechanism of unmasking of the second tripeptide site was not assessed, this finding and our results suggest that, under certain conditions, a second site can be filled by either oxytocin or vasopressin or also the tripeptides analogs. In the case of (Lys9) vasopressin it is clear that this capacity is related to the degree of dissociation of the protein dimer, although in the case of oxytocin or tripeptide this may be related to conformational rearrangements of the protein consecutive to LiCl addition or covalent attachment of a synthetic polypeptide.

In conclusion, the above reported observations suggest strongly that the dimeric protein-hormone complex is probably the physiologically operative form in the transport process. Nothing is known about how protein dimers are organized in the neurosecretory granule environment. The above described allosteric properties and half-site reactivity of the system might suggest the possible existence in vivo of a still unknown biological process.

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THEORETICAL APPENDIX

In this appendix we propose to establish the mathematical expressions allowing a quantitative analysis of the experimental data in function of the proposed model.

In the case of a monomer – dimer equilibrium coupled to ligand binding the saturation fraction \( \hat{f} \) can be expressed as the sum of the saturation fractions of the two species weighted by their concentrations. A general form of this expression is:

\[
\hat{f} = f_m \hat{f}_m + f_d \hat{f}_d
\]

where \( f_m \) and \( f_d \) are the fractions of protomers in the monomer and dimer, respectively. In the proposed model for hormone binding to neurophysin, assumptions are made on two classes of independent sites per protomer: (a) one class of sites exhibiting a higher affinity for the ligand in the dimer than in the monomer (\( K_2 \) and \( K_1 \)) are the respective affinity constants and \( K_2 > K_1 \); (b) another class of sites in which occupation by the ligand is in competition with the association areas of the monomer (the association constant is \( K_3 \) in this case). \( X_0 \) is the intrinsic dimerization constant and the definition of an apparent dimerization constant, \( X \), as a function of the free ligand concentration, is given by:

\[
X = \frac{D}{M^2} = \left[ \frac{D + DL + D_L}{M + ML + LM + ML_L} \right] = X_0 \left[ \frac{1 + K_3 L}{1 + K_2 + K_3 L + K_4 L} \right]^2
\]

where \( M \) and \( D \) are, respectively, the sum of all the monomeric and dimeric species. The dimerization state can be evaluated by measurement of the weight average molecular weight \( M_\text{w} \) defined as:

\[
M_\text{w} = M_1 \frac{1 + 4XM}{1 + 2XM} \]

where \( M_1 \) is the molecular weight of the monomer. On the other hand the total concentration of monomer \( M \) can be evaluated from the mass conservation equation of the protein:

\[
2XM^2 + M - C_T \frac{M}{M_1} - O
\]

\( C_T \) is the weight concentration of protein. Thus:

\[
M = \sqrt{\frac{1 + 8X}{4X} C_T M_1 - 1}
\]

\( f_m \) and \( f_d \) the fractions of protomer as monomer or dimer, respectively, can be defined as:

\[
f_m = \frac{M}{M + 2D} = 2 - \frac{M_\text{w}}{M_1}
\]

\[
f_d = \frac{2D}{M + 2D} = \frac{M_\text{w}}{M_1} - 1
\]

The general formulation of the saturation function \( i \), relative to the presented model, as a function of free ligand becomes:

\[
\frac{1}{i} = \frac{2 - f_m}{M_1} \left[ \frac{K_1}{1 + KL} + \frac{K_3}{1 + K_2 L} + \frac{M_\text{w}}{M_1} - 1 \right] \frac{K_2 L}{1 + K_4 L}
\]

In order to determine the parameters \( K_1 \), \( K_2 \), and \( K_3 \) by a nonlinear least squares procedure, we have used in the Scatchard representation an analytical polar expression of the module \( \rho \) (distance between the origin and any experimental point) function of the angle \( \phi \ ((\phi = 1/L) \). This analytical expression shows a higher sensitivity to cooperative phenomenon for low saturation fraction. In such a formulation equation \( \phi \) becomes:

\[
\rho = \frac{\sqrt{2 - f_m}}{M_1} \left[ \frac{K_1}{\sin(\phi + K_1 \cos \phi)} + \frac{K_3}{\sin(\phi + K_4 \cos \phi)} \right] \frac{M_\text{w}}{M_1} \left[ \frac{K_2 L}{\sin(\phi + K_2 \cos \phi)} \right]
\]

Analysis of the experimental Scatchard plots was then performed using expression \( \phi \) by an iterative nonlinear least squares procedure (34) on a Wang 2200 B calculator.

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