COOPERATIVE BINDING OF OCYTOCIN TO BOVINE NEUROPHYSIN

A comparison of two theoretical models

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1. Introduction

The in vitro formation of specific complexes between the 10 000 dalton brain protein, neurophysin, and the neurohypophyseal peptide hormones (ocytocin and vasopressin), constitutes an interesting system for the understanding at the molecular level of both the mechanism by which a peptide can bind a protein molecule and of the biological function of this class of proteins [1–12]. It is now well established that the bovine neurophysins I [8] and II [2,8] tend to self-associate in aqueous solution. From sedimentation equilibrium studies at pH 5.60, I 0.1, values of apparent weight average molecular weight as a function of total protein concentration (in the range 0.05–4 mg/ml) were shown to be consistent with a model specifying that protein solutions comprised reversible equilibrium mixtures of monomer and dimer governed by a single equilibrium association constant $X_0 = 5.8 \times 10^3\ M^{-1}$ [8]. The addition of peptide hormones imposes a constraint on this equilibrium toward the dimeric form of the protein [8]. The Scatchard analysis of the binding isotherms indicated a number of features:

(i) The apparent existence of 1 ocytocin site/10 000 daltons.

(ii) The curvilinearity of the isotherm showing evidence for a low degree of positive cooperativity.

(iii) The clear dependence upon protein concentration of the average slope of the binding isotherms [8].

These results, together with the equilibrium sedimentation studies, led us to propose a model in which the dependence of binding on protein concentration (in the range 5×10^{-6} to 3×10^{-4} M) was explained by assuming a 5-fold greater affinity of ocytocin for each dimer site than for the monomer site [8]. The essential features of the process were confirmed [9,12]. The theoretical curves obtained from this model (model 1), gave good results for the protein concentration dependence of the average slopes of the binding isotherms. However, for low $\bar{\nu}$ values, attempts to get the best curve fit lead to rather poor results suggesting that the cooperativity associated with polymerization cannot be accounted for by the curvature observed at $\bar{\nu} < 0.30$. Hence, curvilinearity observed at low $\bar{\nu}$ values might reflect positive cooperative interactions between the two sites of the dimer. To test for this possibility, this former model (model 1) was revised and further extended to a general monomer $\rightleftharpoons$ dimer equilibriun with 1 site/monomer and 1 site on each protomer of the dimer available for ocytocin, including the possibility of cooperative interactions between the two dimeric sites. We show that this general model (model 2) allows a better fit for the entire binding isotherm at each of the three neurophysin concentrations utilized, than the former one. This can be shown to support the hypothesis of a positive cooperativity within the dimeric sites at pH 5.60, I 0.1.

2. Materials and methods

2.1. *Neurophysin*

Highly purified bovine neurophysin II was prepared and tested for homogeneity using gel electrophoresis, gel isoelectric focusing and amino acid composition as in [3,8].

2.2. *Ocytocin*

Ocytocin was a generous gift of Sandoz (Basel). The tritiated hormone (30 Ci/mmol) was also prepared as reported and routinely tested for its radiochemical purity by the usual electrophoretic and chromatographic tests [3,8].

2.3. *Equilibrium dialysis*

Ocytocin binding studies were run at 24 ± 1°C, pH 5.60 (0.1 M Na-acetate buffer, I 0.1) as detailed [3,6,8]. Refined analysis of the Scatchard plots required that a minimum of 90% of the entire saturation curve be tested (from 5×10^{-7} M to 10^{-3} M) at each protein concentration (5×10^{-6} M, 5×10^{-5} M and 3×10^{-4} M). Each point was run in quadruplicate. Concentrations of neurophysin II and ocytocin were evaluated both by dry weight and spectrophotometrically on a Cary 118 C Spectrophotometer. Fractional saturation ratios $\bar{\nu}$ were expressed as bound ligand concentrations/protein molar concentration considering monomer mol. wt 10 041.

2.4. *Fittings*

Computations were performed on the IBM 970/168 at the Centre Interregional de Calcul Electronique (ORSAY).

3. Results

In the case of a monomer $\rightleftharpoons$ dimer equilibrium modulated by ligand binding the saturation fraction $\bar{\nu}$ can be expressed as the sum of the saturation fractions of the two species weighted by the fraction of protomers in the monomer and dimer, respectively:

$$\bar{\nu} = f_M \cdot \bar{\nu}_M + f_D \cdot \bar{\nu}_D \qquad (1)$$

where f_M and f_D are the fractions of protomers in monomer and dimer, respectively. In the general proposed model for ocytocin binding to neurophysin (fig.1), assumptions are made on the existence of

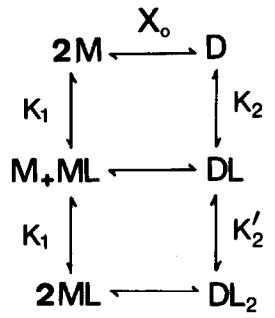

Fig.1. A general schematic model (model 2) of the binding of ocytocin to bovine neurophysin II at pH 5.60. M is the monomer, D is the dimer and L is the ligand (ocytocin). X_0 is the dimerization constant for the unliganded protein and K_1, K_2, K'_2 are the intrinsic association constants of the described equations. Note that this model might be simplified to the case of two equivalent and independent dimeric binding sites by assuming $K_2 = K'_2$ (model 1).

(i) 1 site/monomer (K_1 is the intrinsic association constant).

(ii) 1 site/protomer of the dimer with two different intrinsic association constants K_2 and K'_2 for the first and second dimeric sites, respectively.

(iii) In the absence of ligand, X_0 is the intrinsic dimerization constant ($X_0 = D/M^2 = 5.80 \times 10^3$ M where M and D are the molar concentrations of monomer and dimer, respectively.

The definition of an apparent dimerization constant X, as a function of the free ligand concentration, is given by:

$$X = \frac{\bar{D}}{\bar{M}^2} = \frac{[D + DL + DL_2]}{[M + ML]^2}$$

$$= X_0 \frac{[1 + 2K_2L + K_2K'_2L^2]}{[1 + K_1L]^2} \qquad (2)$$

where $\bar{M}$ and $\bar{D}$ are, respectively, the sum of all the monomeric and dimeric species [13]. The dimeriza-

tion state can be defined by the weight average molecular weight as:

$$\bar{M}_w = \frac{M_1^2 \bar{M} + 4M_1^2 \bar{D}}{M_1 \bar{M} + 2M_1 \bar{D}}$$

$$= \frac{\bar{M} + 4\bar{D}}{\bar{M} + 2\bar{D}} = M_1 \frac{1 + 4X\bar{M}}{1 + 2X\bar{M}} \quad (3)$$

where M_1 is the molecular weight of the monomer.

On the other hand, the total concentration of monomer $\bar{M}$ can be evaluated from the mass conservation of the protein:

$$2X\bar{M}^2 + \bar{M} - C_T / M_1 = 0 \quad (4)$$

C_T is the weight concentration of protein. Thus:

$$\bar{M} = \frac{\sqrt{1 + 8X C_T / M_1} - 1}{4X} \quad (5)$$

In eq. (1), f_M and f_D can be defined as:

$$f_M = \frac{\bar{M}}{\bar{M} + 2\bar{D}} = 2 - M_w/M_1$$

$$f_D = \frac{2\bar{D}}{\bar{M} + 2\bar{D}} = M_w/M_1 - 1 \quad (6)$$

and $\bar{\nu}_M$ and $\bar{\nu}_D$ as:

$$\bar{\nu}_M = \frac{K_1 L}{1 + K_1 L}$$

$$\bar{\nu}_D = \frac{K_2 L + K_2 K_2' L^2}{1 + 2K_2 L + K_2 K_2' L^2} \quad (7)$$

$\bar{\nu}_M$ and $\bar{\nu}_D$ reflect the saturation of one site on the monomer and of two interacting sites on the dimer, respectively. The general formulation of the saturation function $\bar{\nu}$ eq. (1), relative to the presented general model, as a function of free ligand becomes:

$$\bar{\nu} = [2 - M_w/M_1]\left[\frac{K_1 L}{1 + K_1 L}\right] + [M_w/M_1 - 1]\left[\frac{K_2 L + K_2 K_2' L^2}{1 + 2K_2 L + K_2 K_2' L^2}\right] \quad (8)$$

In the case of two equivalent and independent sites on the dimer, eq. (8) can be simplified assuming $K_2 = K_2'$ (model 1).

In order to determine the optimized parameters K_1, k_2, and K_2' from the experimental points by a nonlinear iterative least squares procedure [14], we used in the Scatchard representation an analytical pola expression as in [8]. To take in account the uncertainty in the concentration measurements, we introduced in the fit the number of sites per protomer as a fourth unknown parameter. Figure 2 shows the experimental results and fits assuming the equivalence and independence of the two dimeric sites of the first model (model 1). Figure 3 shows identical experimental results and the fits based on the general model (model which assumes a positive cooperative interaction

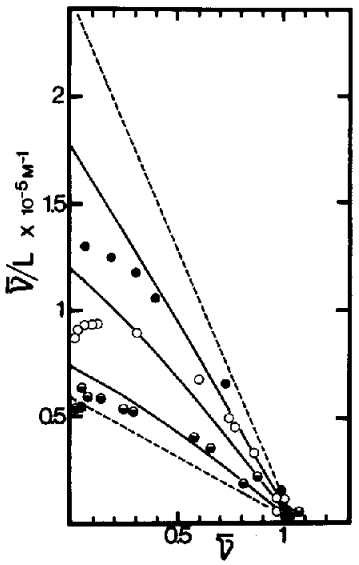

Fig.2. Scatchard plots of the binding of ocytocin (from 5×10^{-7} M to 10^{-3} M) in 0.1 M Na-acetate buffer, I 0.1, pH 5.60, at 24 ± 1°C, to various concentrations of neurophysin II: (● ● ●) neurophysin II, 5×10^{-6} M; (○ ○ ○) neurophysin II, 5×10^{-5} M; (● ● ●) neurophysin II, 3×10^{-4} M. The lines are the best fitting curves based on model 1 (see text and table 1). The dashed lines are theoretical relationships based on model 1 assuming both an infinite and a zero limit protein concentration range.

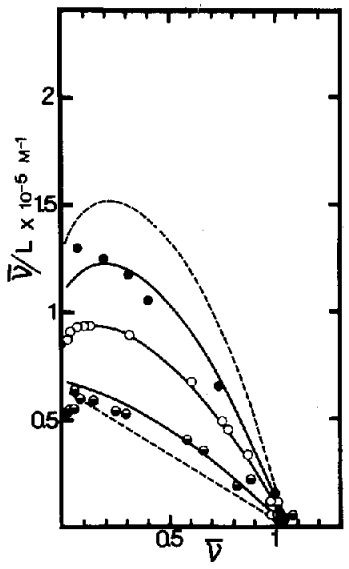

Fig.3. Scatchard plots of the binding of ocytocin (from 5×10^{-7} M to 10^{-3} M) in 0.1 M Na-acetate buffer, I 0.1, pH 5.60, at 24 ± 1°C, to various concentrations of neurophysin II: (⊖ ⊖ ⊖) neurophysin II, 5×10^{-6} M; (○ ○ ○) neurophysin II, 5×10^{-5} M; (● ● ●) neurophysin II, 3×10^{-4} M. The solid lines are the best fitting curves based on model 2 (see text and table 1). The dashed lines are theoretical relationships based on model 2 assuming an infinite and a zero limit protein concentration range.

between the two protomeric sites of the dimer. The four parameters, K_1, K_2, K_3 and n (number of sites) with standard errors, have been determined from these fits and are presented in table 1 for each of the two proposed models.

4. Discussion

In the former model of binding that we have previously described (model 1) ([8] and above results) it was assumed, in order to simplify the equations, that the two dimeric sites were equivalent and independent, i.e., that the hormone did not induce isomerization of the dimeric species. Theoretical Scatchar curves drawn according to this simple model gave a good fit for the concentration dependence of the average isotherms by assuming a 5-fold greater affinity of ocytocin for each equivalent dimer site than for the corresponding monomer site (see fig.2 and table 1). However, this former model does not fit well the general shape of the experimental isotherms, especially at low $\bar{\nu}$ values (fig.2).

The second model (model 2), complicated by introducing additional equations accounting for the possible pre-existant, or ligand-induced, isomerization of the dimer, indicates a more satisfactory fitting with our experimental binding curves (see fig.3 and table 1). In other words, such a model tells us that occupancy of the first site on the neurophysin dimer gives a 4-fold increase in the intrinsic affinity for the second hormone molecule bound. Despite the fact that the ligand-induced isomerization model is more probable than the pre-existing one [4,6,9,10], there are no definitive experimental means of distinguishing between them and it would appear that a clear cut choice between these two hypotheses is extremely hazardous at this time. However, it should be noted that a number of experimental data [8] providing the basis for these models have been already confirmed

Table 1

	K_1 (M^{-1})	K_2 (M^{-1})	K'_2 (M^{-1})	n
Model 1	$5.94 \pm 0.64 \times 10^4$	$2.42 \pm 0.18 \times 10^5$ RMS[a] = 0.0345	$2.42 \pm 0.18 \times 10^5$	1.06 ± 0.01
Model 2	$6.11 \pm 0.50 \times 10^4$	$1.30 \pm 0.23 \times 10^5$ RMS[a] = 0.0260	$5.35 \pm 0.10 \times 10^5$	1.04 ± 0.09

[a]RMS (residues means square) = $\dfrac{\Sigma\,(\phi\ \text{exp.} - \phi\text{theor.})^2}{(N-4)}$

Values for the four optimized parameters K_1, K_2, K'_2 and n deduced from the experimental binding points by an iterative non-linear least square procedure based on model 1 (two equivalent and independent dimeric sites) and model 2 (cooperative interaction between the two dimeric sites), respectively

by observations from various authors, especially concerning

(i) The protein concentration dependence for the apparent equilibrium association constant [9].
(ii) The higher affinity (5-fold) of ocytocin for the neurophysin dimer than for the monomer [9].
(iii) The predominance of dimeric complexes under normal binding conditions [12].

The discrepancies between our dimeric association constants ratio values ($K_2'/K_2 = 4$) and those in [10] about the binding of ocytocin to neurophysin II at pH 5.8, or those in [9] ($K_2'/K_2 = 2$) about the binding of the dipeptide Phe–Tyr–NH_2 to mononitrated neurophysin II at pH 6.2, provide a further illustration of the observation that it may be rather innacurate to consider a restricted protein concentration range when analyzing results in terms of a particular model, especially a polymerizing one. Since a fit obtained with a single curve does not permit a reliable determination of all the unknown parameters and since such a fit ignores the monomer–dimer equilibrium modulated by ligand binding, caution should be taken in its interpretation.

We therefore conclude that the binding of ocytocin to neurophysin can be described as follows:

(i) Ocytocin binding enhances significantly the formation of dimeric species because of a greater affinity for the dimer sites than for the monomer site.

(ii) There are, respectively, 1 site/monomer ($K_1 = 5–6 \times 10^4\ M^{-1}$) and 1 site/protomer on the dimer.

(iii) There is a positive cooperative interaction between these two sites on the dimer such that binding to the first site ($K_2 = 1.3 \times 10^5\ M^{-1}$) on the dimer quadruple the affinity for the second ligand molecule bound ($K_2' = 5.3 \times 10^5\ M^{-1}$).

A model in which $\gamma = 0$ (γ is the number of binding sites on M) was tested and gave significantly different ordinate intercepts from the ones we experimentally obtain suggesting that binding on the monomer probably occurs to some extent ($k_1 = 6 \times 10^4\ M^{-1}$).

It should be noted that the revised polymerization–isomerization model share some of the qualitative features suggested by the first model [8] about the still unknown exact physiological role of neurophysins [15].

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