Co-operative Binding of Oxytocin to Bovine Neurophysin II

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The interaction of oxytocin with bovine neurophysin II in 0.1 M-sodium phosphate, pH 5.8, was investigated by equilibrium-dialysis and sedimentation studies. Sigmoidality of the binding curve is attributed to isomerization, either hormone-induced or pre-existing, with preferential binding of oxytocin to one isomeric state. Results are consistent with a binding equation of the form $r = (2P[S] + 2PQ[S]^2)/(1 + 2P[S] + PQ[S]^2)$ and values of $0.7 \times 10^5 \text{ M}^{-1}$ and $1.3 \times 10^5 \text{ M}^{-1}$ for P and Q respectively. The significance of these two parameters in relation to current theories of allostery is also discussed.

Neurophysins bind the neurohypophyseal hormones oxytocin and vasopressin with a high degree of selectivity (Acher *et al.*, 1958). Quantitative studies of these protein-hormone interactions by equilibrium dialysis have been interpreted in terms of linear Scatchard (1949) plots and the presence of one oxytocin-binding site per base mol (10000 g) of neurophysin II (Breslow & Abrash, 1966; Breslow & Walter, 1972; Camier *et al.*, 1973). However, recent data on the neurophysin-vasopressin interaction (Pliška & Sachs, 1974) exhibited sigmoidality, which was attributed, without proof, to non-equivalence of hormone binding to different polymeric states of neurophysin. In contrast with the earlier studies of the oxytocin-neurophysin II system, we also detect co-operative binding of hormone. The aim of this communication is to present the binding data, together with ultracentrifuge studies to determine the nature of the neurophysin oligomers responsible for the sigmoidal effect.

Materials and methods

Materials. Acetone-dried powder of bovine posterior pituitary lobes was supplied by Paines and Byrne Ltd., Greenford, Middx., U.K., and a sample of [^3H]oxytocin (Sjöholm & Carlsson, 1967) was kindly given by Dr. I. Sjöholm, Farmakognostika Institutionen, Stockholm, Sweden. Dialysis membranes (Visking tubing 18/32) were purchased from the Scientific Instrument Centre Ltd., London W.C.1, U.K. Sephadex products (G-25, G-75 and DEAE-Sephadex A-25) were obtained from Pharmacia (G.B.) Ltd., London W.5, U.K., and reagent-grade chemicals were from BDH Chemicals Ltd., Poole, Dorset, U.K.

Neurophysin-hormone complex was prepared from the acetone-dried powder of bovine posterior

pituitary lobes by the method of Chauvet *et al.* (1960). Hormone-free protein was then obtained by gel chromatography in 0.1 M-formic acid (Frankland *et al.*, 1966; Hollenberg & Hope, 1968). The resulting crude neurophysin was fractionated further by ion-exchange chromatography on DEAE-Sephadex A-25 in Tris-HCl (I 0.05 M, pH 8.1) with a salt gradient (0–300 mM-NaCl) created by an LKB Ultrograd 1300 gradient mixer (LKB-Produkter AB, Bromma, Sweden). The electrophoretically homogeneous samples of neurophysins I, II and C were desalted by dialysis and then freeze-dried.

N-Benzoyloxycarbonyl-SS'-dibenzylxytoceine was synthesized by the stepwise *p*-nitrophenyl ester method (Bodanszky & du Vigneaud, 1959) and converted into the cyclic peptide as described previously (Wälti & Hope, 1973). This was mixed with a sample of [^3H]oxytocin and purified by counter-current distribution in a fully automatic train of 200 tube-units (H. O. Post and Co., Maspeth, N.Y., U.S.A.). After 700 transfers in the solvent system butan-1-ol-propan-1-ol-0.05% acetic acid (2:1:3, by vol.), biologically active material (526 ± 71 i.u./mg; isolated rat uterus) with a specific radioactivity of 14.087 mCi/mmol concentrated in a single peak (K 0.36) and was isolated as a freeze-dried analytically pure peptide.

Equilibrium dialysis. Binding experiments were carried out at $22 \pm 2^\circ\text{C}$ with solutions (1 ml of each) of bovine neurophysin II (1.05 ± 0.09 mg/ml) and [^3H]oxytocin ($13\text{--}750 \mu\text{g/ml}$) in 0.1 M- NaH_2PO_4 , pH 5.8. Dialysis membranes were pretreated by soaking in 0.05% potassium ferricyanide solution at 2°C for 48 h, followed by successive washes with tap water, distilled water and finally sodium phosphate buffer. Before use the membranes were placed in the 0.1 M-phosphate, pH 5.8, for 24 h to ensure equilibration of the membrane with buffer. Modified

Myer & Schellmann (1962) dialysis cells consisting of cylindrical two-compartment polymethacrylate cells with a maximum volume of 1.3 ml per half-cell were used for these experiments. A period of 36–40 h was allowed for the solutions to attain dialysis equilibrium. Concentrations of bound and free ligand were determined by direct scintillation counting of 10–50 μ l portions in 5 ml of Bray's (1960) solution on a Beckman LS-200B liquid-scintillation counter.

Sedimentation. Ultracentrifuge experiments were conducted at 20°C in a Spinco model E apparatus. The resulting schlieren patterns were measured on a Nikon two-dimensional comparator fitted with projection screen and accurate to 2 μ m. Equilibrium sedimentation patterns from centrifugation at 20000 rev./min of 1 mm columns of neurophysin (1.5–5.0 mg/ml) in 0.1 M- NaH_2PO_4 , pH 5.8, were analysed by the Lamm (1929) procedure: the partial specific volume was taken as 0.71 (Hollenberg & Hope, 1967). The effect of oxytocin on the sedimentation of bovine neurophysin II was tested by velocity experiments at 60000 rev./min in which synthetic boundaries were formed between neurophysin solutions (1 mg/ml) containing known amounts of oxytocin and solutions containing the corresponding equilibrium concentrations of hormone: the latter could be estimated from the binding curve, which was constructed from experiments with the same neurophysin concentration. Since the sedimenting boundaries were essentially symmetrical, s_{20} values were determined from the rates of migration of the schlieren maxima.

Results and discussion

The Scatchard (1949) plot of equilibrium-dialysis experiments on the binding of oxytocin to bovine neurophysin II at pH 5.8 is shown in Fig. 1, about which the following points require comment. (1) The binding ratio, r (Klotz, 1946), is expressed in terms of dimeric neurophysin, which has a molecular weight of 20082 based on amino acid composition (Breslow *et al.*, 1971; Walter *et al.*, 1971). Low-speed equilibrium-sedimentation studies of neurophysin solutions with initial concentrations in the range 1.5–5.0 mg/ml yielded linear Lamm plots and a z -average molecular weight of 20000 ± 200 , a result that compares favourably with investigations at pH 4.8 (Hollenberg & Hope, 1967). (2) Binding data for higher concentrations of free ligand ($r > 0.4$) are in very good agreement with earlier equilibrium-dialysis studies. Association constants of $1.4 \times 10^5 \text{ M}^{-1}$ (Breslow & Abrash, 1966, as re-interpreted by Breslow & Walter, 1972) and $0.4 \times 10^5 \text{ M}^{-1}$ (Camier *et al.*, 1973) have been reported, whereas a value of $0.95 \times 10^5 \text{ M}^{-1}$ would be inferred from the broken line in Fig. 1, which represents the linear relationship obtained by regression analysis of data with $r > 0.4$. (3) At lower

concentrations of unbound hormone ($r < 0.4$) the broken line represents a very poor fit of the experimental results. Although binding data at either extremity of a Scatchard plot are less precise, the error bars in Fig. 1 denote the estimated maximum uncertainty in each experimental point, and hence the downward curvature at low r is considered to reflect a real phenomenon. Such curvilinearity is characteristic of sigmoidal binding (Frieden & Colman, 1967; Changeux *et al.*, 1968).

Because of the numerous theoretical models that can give rise to sigmoidal binding curves (see, e.g., Nichol & Winzor, 1972), sedimentation-velocity runs on neurophysin in the presence and absence of oxytocin were performed to determine the oligomeric state of neurophysin in the protein-hormone complex. The sedimentation coefficient (s_{20}) was 2.0 S for neurophysin alone and 2.1 S for neurophysin saturated ($r > 1.98$) with oxytocin. Since the binding of hormone causes a 10% increase in the molecular weight of neurophysin dimer, the results from velocity experiments show that neurophysin exists normally as a dimer and dimerization is not induced by the ligand. This suggests that isomerization, either pre-existing (Monod *et al.*, 1965) or hormone-induced (Koshland *et al.*, 1966), is producing the sigmoidal binding curve.

Since we have no experimental means of distinguishing between the models of Monod *et al.* (1965) and Koshland *et al.* (1966), we have decided to express

Fig. 1. Scatchard plot of binding data for the interaction of oxytocin with bovine neurophysin II in 0.1 M-phosphate, pH 5.8

Bars denote the extent of the estimated experimental error associated with each point. The lines are theoretical relationships based on eqn. (5) with $P = Q = 0.95 \times 10^5 \text{ M}^{-1}$ (----), and $P = 0.7 \times 10^5 \text{ M}^{-1}$, $Q = 1.3 \times 10^5 \text{ M}^{-1}$ (—).

the sigmoidality in a form consistent with either theory. The former model of the present system may be written as

where X denotes the equilibrium constant for the isomerization of neurophysin, and K_A and K_C the respective intrinsic association constants (Klotz, 1946) for the binding of oxytocin to the two equivalent and independent sites on the isomeric states A and C. The binding equation for this system is usually expressed as

$$r = \frac{2K_A[S](1 + K_A[S])}{(1 + K_A[S])^2} + \frac{2XK_C[S](1 + K_C[S])}{X(1 + K_C[S])^2} \quad (2a)$$

where $[S]$ denotes the equilibrium concentration of oxytocin, but we choose to rearrange eqn. (2a) as

$$r = \frac{2[S](K_A + XK_C) + 2[S]^2(K_A + XK_C)(K_A + K_C)}{1 + 2[S](K_A + XK_C) + [S]^2(K_A + XK_C)(K_A + K_C)} \quad (2b)$$

In simplest form the ligand-induced isomerization model may be represented as

where K_1 denotes the association constant for the binding of oxytocin to either of the equivalent sites on neurophysin, and Y the equilibrium constant for the isomerization of AS that leads to stronger binding ($K_2 > K_1$) of hormone to the remaining site. For this model the binding equation is

$$r = \frac{2[S](K_1 + YK_1) + 2[S]^2(K_1 + YK_1)[K_2Y/(1 + Y)]}{1 + 2[S](K_1 + YK_1) + [S]^2(K_1 + YK_1)[K_2Y/(1 + Y)]} \quad (4)$$

Eqns. (2b) and (4) are of analogous form, i.e.

$$r = \frac{2P[S] + 2PQ[S]^2}{1 + 2P[S] + PQ[S]^2} \quad (5)$$

with the only difference the interpretation placed on the constant coefficients P and Q . The solid line in Fig. 1, which represents eqn. (5) with $P = 0.7 \times 10^5 M^{-1}$ and $Q = 1.3 \times 10^5 M^{-1}$, is considered to describe the experimental results better than the broken line ($P = Q = 0.95 \times 10^5 M^{-1}$). Although even greater curvature at low r values seems indicated by the experimental data, attempts to curve-fit this region more precisely lead to poorer correspondence

between theory and experiment at higher r values. We therefore conclude that the binding of oxytocin to neurophysin is a co-operative phenomenon involving isomerization of dimeric neurophysin or neurophysin-hormone complex, and that there is a factor of approx. 2 between the coefficients P and Q in the expression of the binding equation as the ratio of two polynomials (eqn. 5).

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