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3′:5′-Cyclic Adenosine Monophosphate Phosphodiesterase: Negative Cooperativity

(kinetics/models/computer analysis/enzyme regulation)

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ABSTRACT **Kinetic and chromatographic analysis of cyclic nucleotide phosphodiesterase (EC 3.1.4.c) obtained from rat tissue has revealed that this enzyme exists in at least two molecular forms. After chromatographic separation, one form (cyclic AMP phosphodiesterase) still exhibits kinetics suggestive of the action of either two enzymes or one enzyme under negative cooperative regulation. Computer model studies were undertaken to distinguish between these two possibilities. The matrix method was used to generate the partition functions for (*a*) the sum of two independent enzymes and (*b*) one enzyme exhibiting negative cooperative kinetics. The experimental data were fitted to the theoretical models by a nonlinear least-squares computer program. The results show that, while both models can fit the data, the two-enzyme model would require contamination far in excess of what is detectable physically or by activity measurements. Thus, the negative cooperative model seems the more appropriate theoretical explanation of the observed kinetic behavior of this enzyme. The implication of negative cooperativity with respect to the regulation of cyclic AMP concentrations in physiological systems is discussed.**

Cyclic nucleotide phosphodiesterase (EC 3.1.4.c.) has been found in numerous mammalian tissues (1–4) and in bacteria (5, 6) since its discovery by Sutherland and Rall in dog liver (7). Kinetic studies have suggested that more than one cyclic nucleotide phosphodiesterase exists (8). Results from this laboratory (9, 10) and others (11–14) have shown by various separation procedures that at least two distinct enzymatic activities are present in these tissues. Of the two major active fractions eluted from Agarose gel columns, one (fraction II) has an approximate molecular weight of 400,000 and a higher affinity for cyclic GMP (cGMP) than for cyclic AMP (cAMP). The other (fraction III) has a lower molecular weight and displays a higher affinity and specificity for cAMP. After further purification, the phosphodiesterase of fraction III exhibits anomalous kinetics suggestive of either negative cooperativity, as defined by Koshland (15, 16), or the combined action of two Michaelis–Menten enzymes. Computer model studies were undertaken to determine which of the above mechanisms is most consistent with the experimental data.

MATERIALS AND METHODS

[*8*-^{3}H]cAMP (specific activity 16.3 Ci/mmol) was obtained from Schwarz BioResearch, Inc. and purified by thin-layer chromatography on cellulose. cAMP was purchased from Schwarz and not further purified. Biogel A1.5m (6% Agarose) and Sephadex G-200 are products of Biorad Inc. and Pharmacia, respectively. Snake venom (*Ophiophagus hannah*) was obtained from Sigma Chemical Co.

The cyclic nucleotide phosphodiesterases used in these studies were prepared by the method of Thompson and Appleman (9). The given tissue was homogenized (1:6 w/v) and the pH was adjusted with 1 M acetic acid to 6.0. After sonic disruption with a Biosonic III (30 sec/ml), the solution was centrifuged at 20,000 × *g* for 20 min. About 6 ml of the supernatant solution was placed on a Biogel A1.5m column (2.2 × 170 cm) and eluted with a 50 mM Tris-acetate buffer (pH 6.0) containing 3.75 mM 2-mercaptoethanol. For elimination of possible trace amounts of other active fractions, fraction III from all tissues, concentrated by ultrafiltration, was again chromatographed on Sephadex G-200. To provide an additional criterion of enzymatic purity, rat kidney phosphodiesterase was chromatographed on DEAE-cellulose columns. Columns (1.7 × 30 cm) were equilibrated with the above buffer, and gradients were from 0–1 M sodium acetate. A 0.5–0.8 M sodium acetate fraction off the column could be used to obtain uncontaminated fraction III enzyme without the need of a gradient.

The enzyme assay (9) involves phosphodiesterase hydrolysis of [^{3}H]cAMP to [^{3}H]5′-AMP, which is further converted to [^{3}H]adenosine by snake venom nucleotidase. Unreacted cAMP is removed by binding to Biorad AG1X-2 resin. The supernatant solution containing [^{3}H]adenosine is then counted by liquid scintillation techniques (9).

Theoretical models

Two Independent Michaelis–Menten Enzymes. In order to calculate the configurational partition function (Q_{total}) of this model, we use the matrix method approach (17) as follows:

$$Q_{\text{total}} = F\ (1,1)\ M^n{}_L \begin{pmatrix}1\\0\end{pmatrix} + (1-F)\ (1,1)\ M^n{}_H \begin{pmatrix}1\\0\end{pmatrix}$$

where Q_{total} expresses the sum over all bound and free enzyme species. (1, 1) and $\begin{pmatrix}1\\0\end{pmatrix}$ are row and column vectors, respectively; M_L and M_H are the statistical weight matrix operators for the low and high molecular weight enzymes, respectively; n is equal to the number of catalytic sites per enzyme molecule; and F is the fraction of low molecular

Abbreviations: cAMP, cyclic AMP; cGMP, cyclic GMP.

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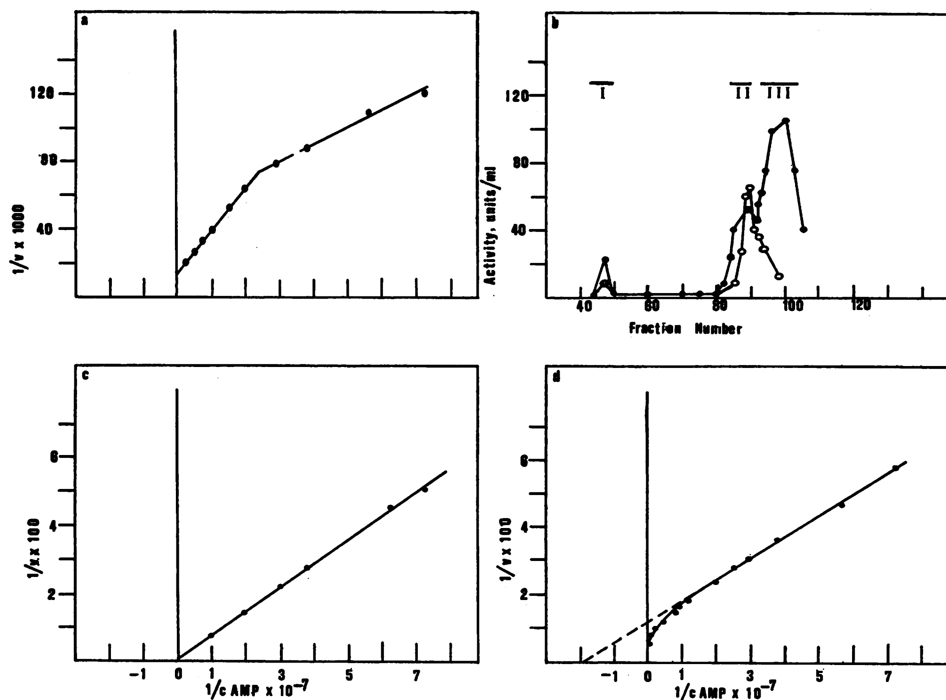

FIG. 1. (*a*) Lineweaver–Burk plot of hydrolysis of [^{3}H]cAMP by a sonicated supernatant preparation from rat brain. (*b*) Agarose gel filtration profile of the preparation described in *a*. ●——●, low cAMP assay (1.25×10^{-7}M); ○——○, high cAMP assay (1.0×10^{-4}M). (*c*) Kinetic analysis of Agarose *fraction II*. (*d*) Kinetic analysis of Agarose *fraction III*.

weight enzyme present. The form of M_L and M_H is

$$M_L = \begin{pmatrix} 1 & 1 \\ mK_L & mK_L \end{pmatrix}; \qquad M_H = \begin{pmatrix} 1 & 1 \\ mK_H & mK_H \end{pmatrix}$$

where m is the concentration of free cAMP, K_L and K_H are the intrinsic association constants to the low and high molecular weight enzymes, respectively.

Negative Cooperativity Model. The total partition function for the negative cooperative model can be written simply as

$$Q_{\text{total}} = (1, 1)\, M^n \begin{pmatrix} 1 \\ 0 \end{pmatrix}$$

where M contains an additional parameter, K_{st}, expressing the degree of cooperativity. For negative cooperativity K_{st} is smaller than unity. For positive cooperativity, $K_{st} > 1$, while for a Michaelis–Menten enzyme, $K_{st} = 1$. The form of M is

$$\begin{pmatrix} 1 & 1 \\ mK & mKK_{st} \end{pmatrix}$$

where K is the intrinsic association constant of a cAMP molecule to the catalytic site.

For either model, the partition function is related to the experimental parameters in the following manner:

$$\theta = (1/n)(\partial \ln Q_{\text{total}}/\partial \ln m)_T$$

where θ represents the fraction of bound enzyme sites, and Q_{total} is the partition function for the given model. The assumption is made that a given change in the reaction velocity is proportional to a change in substrate binding. Thus, it is possible to define θ as the ratio of the reaction velocity (at m) to the maximum velocity. The experimental data are fitted to the model by a nonlinear least-squares computer program (18) similar to that developed by Cornish–Bowden and Koshland (19). The quantity $\Delta\theta$ ($\theta_{\text{experimental}} - \theta_{\text{calculated}}$) is expressed as a Taylor series of the model parameters. When the first derivative of $\Delta\theta$ approaches zero, the error surface approaches a minimum. Thus, the best numerical values of the model parameters are obtained.

RESULTS

A Lineweaver–Burk plot (20) of a sonicated supernatant preparation of rat brain phosphodiesterase shows definite biphasic kinetic behavior (Fig. 1*a*). Similar results are obtained with phosphodiesterase from rat adipose tissue, kidney, heart muscle, and skeletal muscle (10). The form of the curve suggests the action of two enzymes or of one enzyme undergoing negative cooperative regulation. Chromatographic resolution shows that, in fact, two separable activities exist in this preparation (Fig. 1*b*). Hydrolysis of cAMP by fraction II follows normal Michaelis–Menten kinetics (Fig. 1*c*). However, fraction III, consisting of only one peak, still exhibits anomalous kinetics, even after rechromatography on Sephadex G-200.

In order to determine whether the anomalous kinetics is due to contamination of fraction III by fraction II or to negative cooperativity, we did computer model studies. The computer input included the initial velocities and the total concentrations of cAMP. The adjustable parameters for the two-enzyme model are K_L, K_H, and $V_{\max}$; K, K_{st}, and $V_{\max}$ serve as parameters for the negative cooperative model. The results are plotted as $v/V_{\max}$ or θ against the log of the free cAMP concentrations. The best parameter fit for each rat tissue and for rat erythrocyte ghosts was obtained for $n = 2$, i.e., the catalytic site number is taken to be equal to 2 (Fig.

TABLE 1. *Parameters for a negative cooperative model; n = 2*

Rat tissue	K_o*	K_{st}†	Error‡
Brain	7.55 × 10^4	0.244	0.040
Adipose tissue	7.21 × 10^3	0.539	0.018
Kidney	5.58 × 10^4	0.268	0.019
Skeletal muscle	2.28 × 10^4	0.384	0.027
Heart muscle	7.54 × 10^4	0.386	0.020
Erythrocyte ghosts	1.47 × 10^5	0.217	0.050

* K_o = the intrinsic association constant.
† K_{st} = the cooperativity constant.
‡ The error = the average deviation between the experimental data points and the calculated model fit on a scale of 0–1.00.

2). Experimental conditions did not permit the accurate determination of θ values approaching unity due to the low specific activity of cAMP that results at high substrate concentrations with the standard assay. Table 1 gives the intrinsic association constants and the cooperativity constants for the tissues investigated. The cooperativity constants are all less than one, and thus indicative of negative cooperativity. The phosphodiesterase from rat brain and erythrocyte ghosts show the highest degree of negative cooperativity, while that from adipose tissue shows the lowest. Since $n = 2$, the adsorption isotherm is nonsymmetrical with respect to $\theta = 0.5$. The point of inflection of the sigmoid binding curve occurs at higher substrate concentrations for negative cooperative systems. Thus, the reciprocal of the product of the intrinsic association constant and K_{st} is greater than the half-saturation value (21).

Table 2 lists the instrinsic association constants and the fraction of contaminant enzyme (1 − F) for a model representing the sum of two Michaelis-Menten enzymes for the tissues investigated. The amount of contamination by fraction II that is required to give the best parameter fit is greater than 75% in all cases. Experimentally this amount of contamination is extremely unlikely, as the enzyme used in these studies is well separated from fraction II (Fig. 3). The maximum amount of contamination that might be present, as estimated by the possible chromatographic overlap and by the relative amount of cGMP hydrolysis by the highly cAMP-specific fraction III enzyme is 10%. In support of these findings, DEAE-cellulose chromatography of a kidney supernatant preparation also yields two active phosphodiesterase fractions (DII and DIII). Fraction DII hydrolyzes both cAMP and cGMP in a manner similar to Agarose fraction II, while fraction DIII is specific for cAMP hydrolysis, as is Agarose fraction III (Fig. 4*a*). By the criteria of ion-exchange chromatography (Fig. 4*b*), gel filtration (Fig. 4*c*), and kinetic behavior (Fig. 4*d*), these preparations of phosphodiesterase are identical. There is no indication during the separation procedures of contamination by other enzyme species. Based on the above experimental data and theoretical calculations, the negative cooperative model is favored. The instability of this regulatory enzyme to certain conditions has restricted analysis on polyacrylamide gels.

TABLE 2. *Parameters for the sum of two independent enzymes model*

Rat tissues	K_A*	K_B†	(1 − F)‡	Error§
Brain	1.28 × 10^6	2.38 × 10^4	0.84	0.020
Adipose tissue	3.37 × 10^6	5.29 × 10^3	0.97	0.009
Kidney	2.93 × 10^5	1.65 × 10^4	0.77	0.011
Skeletal muscle	5.29 × 10^5	1.15 × 10^4	0.91	0.017
Heart muscle	1.25 × 10^6	3.88 × 10^4	0.91	0.010
Erythrocyte ghosts	2.57 × 10^6	4.14 × 10^4	0.82	0.029

* K_A = the intrinsic association constant to the high-affinity enzyme.
† K_B = the intrinsic association constant to the low-affinity enzyme.
‡ (1 − F) = the fraction of low-affinity contaminant enzyme required to give the best fit.
§ The error = the average deviation between the experimental data points and the calculated model fit on a scale of 0–1.00.

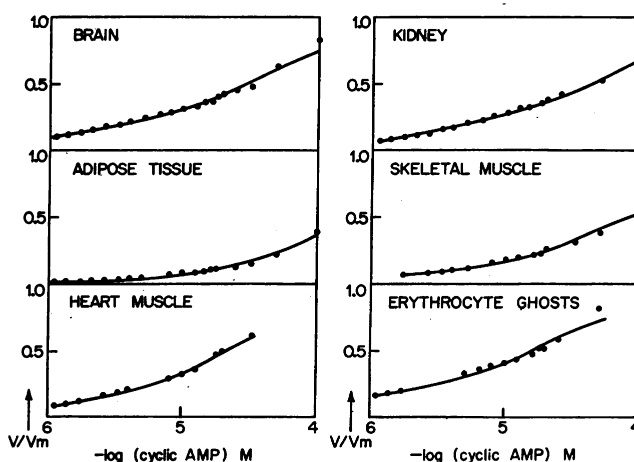

FIG. 2. Plots of θ (V/V_m) against the log of the concentration of cAMP for phophodiesterase activity in preparations from five rat tissues and rat erythrocyte ghosts. The *points* represent the experimental data; the *curves* represent the best computer fit for a negative cooperative model with two catalytic sites.

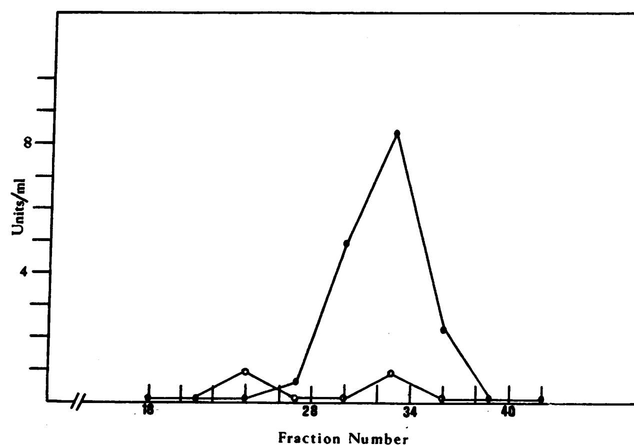

FIG. 3. Sephadex G-200 profile of Agarose fraction III enzyme. ●—●, cAMP units; ○—○, cGMP units (which measure contaminating fraction II enzyme).

FIG. 4. (*a*) DEAE-cellulose profile of a sonicated supernatant preparation from rat kidney. (*b*) DEAE-cellulose profile of rat kidney Agarose-fraction III. (*c*) Agarose gel filtration profile of DEAE-cellulose fraction DIII. The ratio of the elution volume to the void volume is 1.90 on Biogel A0.5m. ●——●, low cAMP assay (1.25×10^{-7}M); ○——○, high cAMP assay (2.0×10^{-5} M). (*d*) Kinetic analysis of DEAE-cellulose fraction DIII. This fraction contains no detectable cGMP hydrolytic activity.

DISCUSSION

Negative cooperativity has been suggested as a mechanism for protection against drastic changes in the chemical environment of an organism (15). Negative cooperative systems can act as kinetic buffers, thus having the opposite effect to positive cooperative systems (22, 23), which may act more like a switch to alter the regulatory state of an organism. The model used in the description of the kinetics of fraction III is visualized in Fig. 5. Since the computer fit gave the best results for n equal to 2, the phosphodiesterase is depicted as having two catalytic sites. Initially both sites are identical with regard to their binding constants. Upon binding of the first molecule of cAMP, site–site or subunit–subunit interactions may cause a configurational modification of the second site, thereby making it more difficult for the second site to bind cAMP. This type of interaction is characterized by an unfavorable (repulsive) energy of interaction and a cooperativity constant less than one. On the other hand, a positive cooperative system is characterized by a favorable (attractive) energy of interaction and a cooperativity constant greater than one. The model we have proposed for cAMP phosphodiesterase is a negative cooperative model in the induced-fit sense, involving interactions between substrate sites. Proof of this model will require direct physical measurements on a highly purified enzyme preparation.

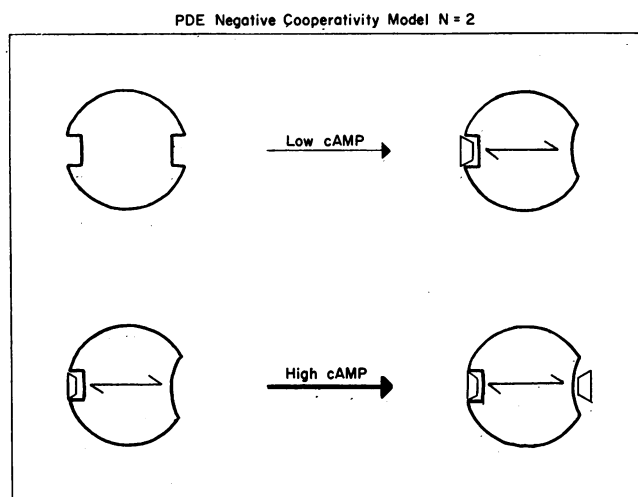

FIG. 5. A hypothetical representation for a two-subunit negative cooperativity model for cAMP phosphodiesterase.

A further interpretation of negative cooperativity is to postulate the existence of more than one binding site on a

single enzyme whose respective binding affinities are different and independent of the degree of saturation with substrate. Such a model should be thermodynamically, but not physically, equivalent to the one used in this work. It should be noted that the allosteric model of Monod *et al.* (23) is not capable of describing *negative* cooperativity.

A model employing positive and negative mechanisms in the regulation of cAMP concentrations can now be described. The adenylate cyclase produces cAMP, which is hydrolyzed by phosphodiesterase using only its high-affinity site at low cAMP concentrations, thus maintaining a steady-state level of cAMP. A hormone acting as a positive effector (switch) activates the adenylate cyclase. The resulting increased concentration of cAMP affects the appropriate systems, such as activation of glycogenolysis, protein synthesis, or secretion. The phosphodiesterase must return this concentration to the basal value. Since the cAMP must exist intact for a finite period of time to perform its activation role, the concentration of cAMP should not be returned to its basal value *too rapidly*. A positive cooperative system cannot achieve this delicate control since its velocity increases too rapidly over a small change in cAMP concentration. Even the unregulated Michaelis–Menten enzyme may have a change in velocity too great to allow cAMP concentrations to remain sufficiently high. A negative cooperative enzyme appears to be capable of performing this task, however, depending on the value of the cooperativity constant. If K_{st} is very much smaller than one, there will not be a significant change in velocity with a change in substrate concentration. Thus, a relatively high steady-state concentration of cAMP will not be sufficiently lowered. If K_{st} is only slightly less than one, the velocity change will be of such a magnitude as to allow the steady-state concentration of cAMP to be high enough and present for a sufficiently long time to perform its activation functions. However, this concentration will not be too high or present long enough to become damaging to the organism. For the above reasons, the K_{st} values of rat tissue phosphodiesterases that are slightly less than one may become physiologically meaningful. cAMP phosphodiesterase, by using negative cooperativity, can therefore act as an extremely sensitive kinetic buffer that protects the organism from prolonged increased concentrations of cAMP, yet allows the cAMP to exist long enough to perform its role.

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