*ON THE MECHANISM OF ACTION OF ADENOSINE 3′, 5′ CYCLOPHOSPHATE**

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Experiments with intact tissues indicate that adenosine 3′,5′ cyclophosphate exerts an important regulatory role in several reactions, including glycogen degradation and synthesis[1-4] and lipolysis.[5] The cyclic nucleotide is formed from ATP together with pyrophosphate by a cyclizing enzyme[6] localized primarily in the cell membrane in nucleated erythrocytes. It is hydrolyzed to 5′ AMP by a phosphodiesterase.[8] In skeletal muscle,[1] diaphragm,[5] heart,[2, 3] and smooth muscle,[9] adenosine 3′,5′ cyclophosphate concentrations increase rapidly two- to threefold under the influence of epinephrine (for a recent review, see ref. 10).

In glycogen degradation, adenosine 3′,5′ cyclophosphate exerts its control in the phosphorylase *b* kinase activation reaction.[11] In glycogen synthesis, the cyclic nucleotide controls the transferase I kinase which converts the glucose 6-phosphate independent (*I*) form of (UDPG: α-1,4-glucan α-4-glucosyl) transferase (EC 2.4.1.11) to the dependent (*D*) form.[12] Both the activation of phosphorylase *b* kinase, and the conversion of the independent (*I*) form of transferase into the dependent (*D*) form can also be achieved in two different ways, namely, through the action of Ca^{++} and a protein factor, or by mild tryptic action.[11, 13]

Krebs *et al.*[14] have described cell-free systems in which phosphorylase *b* kinase is minimally activated in the absence of the cyclic nucleotide. When the cyclic nucleotide is added, a marked activation is obtained.

In a previous report we determined the sensitivity of the transferase I kinase to adenosine 3′,5′ cyclophosphate.[15] In the present report, experiments are described in which the effect of varying the concentration (separately and together) of ATP and Mg^{2+} on the transferase I kinase, as well as the response to adenosine 3′,5′ cyclophosphate, was studied. Under these conditions it was possible to demonstrate *in vitro* an apparent regulatory role for the cyclic nucleotide, a virtual on-off effect.

Kinetic analysis of these experiments may provide information on the mechanism underlying the action of adenosine 3′,5′ cyclophosphate in this system.

Methods.—The effect of adenosine 3′,5′ cyclophosphate on the transferase I kinase system was studied using a relatively crude preparation which contained both the transferase and the kinase and which was prepared in the following manner. Fresh rabbit muscle was homogenized in a Waring Blendor in Tris-HCl, 50 mM; KF, 100 mM; EDTA, 5 mM pH 7.8 (300 ml per 100 gm muscle). The homogenate was centrifuged for 20 min at 12,000 × *g* (2–4°C), the supernatant filtered through glass wool, and the filtrate centrifuged for 3 hr at 78,000 × *g* (4°C). The 78,000 × *g* precipitate was stored at −60°C. After storage for 1–30 days, one pellet, equivalent to approximately 10 gm of muscle, was homogenized in 5 ml glycerophosphate, 8 mM, pH 7.8, containing mercaptoethanol, 50 mM, and incubated for 45 min at 30°C. This incubation solubilized most of the transferase, and the homogenate was then centrifuged at 51,000 × *g* for 45 min. To the clear supernatant, caffeine was added to a concentration of 5 mM to minimize the phosphodiesterase activity.[8]

The transferase I kinase was tested as described in principle by Friedman and Larner,[16] measuring the decrease in transferase I activity upon incubation with ATP and $MgCl_2$. To 0.1 ml of a

solution containing ATP, $MgCl_2$, and adenosine 3′,5′ cyclophosphate at the concentrations indicated below, 0.25 ml of the 51,000 × *g* supernatant was added. The *kinase* reaction was allowed to proceed for 7 min, and was then stopped by adding 0.1 ml EDTA, 100 mM pH 7.0; this brought the EDTA concentration to 20 mM.

The *transferase* I activity was then determined by the incorporation of C^{14}-labeled glucose from UDPG into glycogen as described by Villar-Palasi *et al.*[17] To tubes with 0.1 ml of a solution containing 6.6 mM UDPG, labeled with C^{14} in the glucose moiety (specific activity 12,000 cpm/μmole), 1 mg rabbit liver glycogen, Tris-HCl 50 mM pH 7.8, and EDTA 5 mM pH 7.8, 0.05 ml of the reaction mixture of the transferase I kinase was transferred. In the absence of glucose 6-phosphate only the transferase I activity was determined. In some tubes the total transferase (I + D) activity was determined by including 10 mM glucose 6-phosphate in the text mixture. The tubes were incubated for 15 min at 30°C, and the reaction was stopped by adding 1.0 ml trichloroacetic acid (60 mg/ml), containing 1 mg/ml glycogen and 2 mg/ml LiBr. The protein precipitate was removed, and from an aliquot of the protein-free supernatant, the glycogen was precipitated with 2 vol of 96% ethanol. The radioactive glycogen was washed twice with 66% ethanol. The radioactive samples were counted in a liquid scintillation counter using dioxane-cellosolve solution according to Bruno and Christian.[18] The transferase I kinase is expressed as the decrease in transferase I activity under the assay condition described, i.e., as decrease in cpm incorporated into glycogen compared to control tubes. The control tubes either contained no ATP, or excess EDTA, which prevented the kinase reaction. The two types of controls gave essentially the same results. During the incubation period there were only minor changes in the total transferase activity.

Results.—Requirement for ATP: The apparent K_m for ATP of the transferase I kinase, and the effect of adenosine 3′,5′ cyclophosphate on this constant were investigated. The concentration of 10^{-6} *M* of the cyclic nucleotide which was used here has been previously determined to give maximal stimulation of the transferase I kinase.[15] Figure 1 shows the transferase I kinase activity as a function of varying ATP concentrations at a constant Mg^{2+} of 4 mM. At concentrations of ATP which exceed the Mg^{2+} concentration, inhibition occurs. In the absence of adenosine 3′,5′ cyclophosphate, the inhibition at high ATP concentration is more pronounced. When these results were plotted according to Lineweaver and Burk[19] (Fig. 2), the K_m values for ATP, in the absence and presence of the cyclic nucleotide, obtained by extrapolation of the linear portion of the curve, were 2 and 0.5 mM, respectively. Thus, adenosine 3′,5′ cyclophosphate causes a fourfold shift in the apparent K_m for ATP. The value for the K_m of the transferase I kinase for ATP (in the absence of adenosine 3′,5′ cyclophosphate) is higher than the value reported by Friedman and Larner[16] probably because of an active adenosine triphosphatase in the relatively crude preparation used in these experiments.

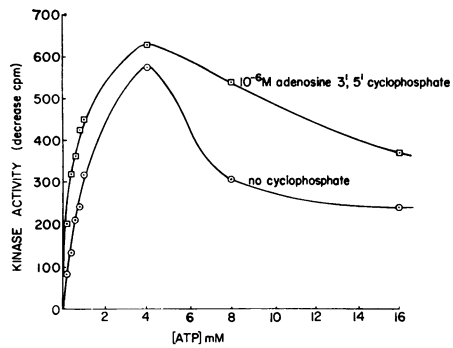

FIG. 1.—Transferase I kinase activity of a rabbit skeletal muscle preparation as a function of ATP concentration with and without adenosine 3′,5′cyclophosphate. Supernatant (0.15 ml of 51,000 × *g*) (see text) containing 5 mM caffeine, 8 mM glycerophosphate pH 7.8, and 50 mM mercaptoethanol was added to 0.10 ml of a solution containing $MgCl_2$ (final conc. 4 mM), ATP, and adenosine 3′,5′ cyclophosphate as indicated. The kinase reaction was allowed to proceed for 7 min at 30°C and stopped by the addition of 0.1 ml EDTA, 100 mM. UDPG: α-1,4-glucan α-4-glucosyl transferase I activity was assayed in a 0.05-ml aliquot by incubation with UDPG and glycogen for 15 min at 30°C.

Figure 3 shows that when the Mg^{2+} concentration is varied at a constant concentration of ATP (8 mM), the transferase I kinase activity both in the absence and in the presence of adenosine 3′,5′ cyclophosphate produced a sigmoidal curve. At low concentrations of Mg^{2+} there was no kinase activity in the absence of adenosine 3′,5′ cyclophosphate, but in the presence of the cyclic nucleotide the activity had a finite value. At higher Mg^{2+} concentrations the cyclophosphate causes a considerable stimulation of the kinase, e.g., at 4 mM Mg^{2+} the stimulation is greater than 100 per cent. A higher concentration of Mg^{2+} (20 mM) causes maximal activity, and adenosine 3′,5′ cyclophosphate has no further effect. When these results were plotted in a double reciprocal plot (Fig. 4), two curved lines were obtained which were concave in the upward direction. The linear portions of the lines with and without adenosine 3′,5′ cyclophosphate have a common intercept with the vertical axis.

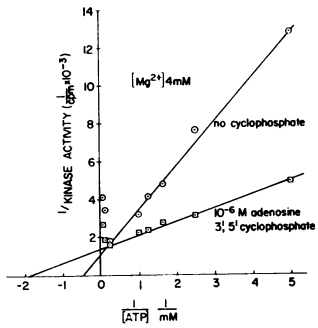

FIG. 2.—Double reciprocal plot of transferase I kinase activity versus ATP concentration with and without adenosine 3′,5′ cyclophosphate. Experimental conditions as in Fig. 1.

When the ATP:Mg^{2+} ratio was kept constant at 2, and the concentrations of both ATP and Mg^{2+} were varied, similar sigmoidal curves and upward curved Lineweaver-Burk plots, not shown here, were obtained. A plot of the inverse of the relative maximal velocity (V/v) against the inverse Mg^{2+} concentration as shown in Figure 5 gave a similar kinetic picture.

Discussion.—The behavior of transferase I kinase in the absence of the cyclic nucleotide is compatible with activation of the enzyme by its cationic cofactor, Mg^{2+}, as outlined for a theoretical case by Dixon and Webb[20] and by Friedenwald and Maengwyn-Davies.[21] It is assumed in the following discussion that the sub-

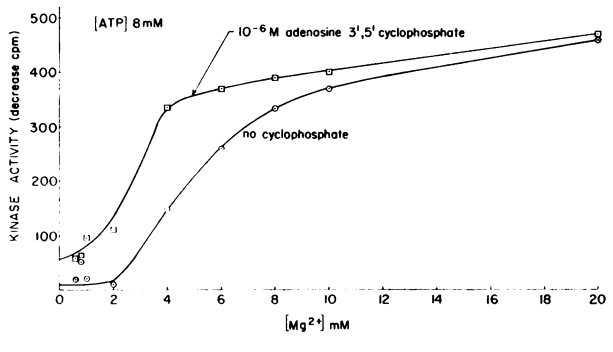

FIG. 3.—Transferase I kinase activity as a function of Mg^{2+} concentration with and without adenosine 3′,5′ cyclophosphate. Supernatant (0.15 ml of 51,000 × g) (see text) containing 5 mM caffeine, 8 mM glycerophosphate pH 7.8, and 50 mM mercaptoethanol was added to 0.10 ml of a solution containing ATP (final concentration 8 mM), $MgCl_2$, and adenosine 3′,5′ cyclophosphate as indicated. The kinase reaction was stopped after a 7-min incubation period at 30°C by addition of 0.1 ml EDTA pH 7.0, and the transferase I was assayed in a 0.05-ml aliquot by incubation with UDPG and glycogen for 15 min at 30°C.

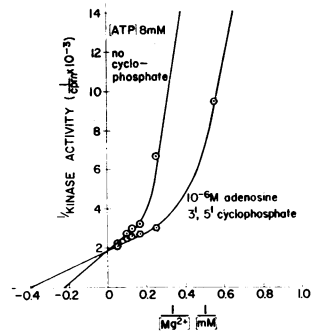

FIG. 4.—Double reciprocal plot of transferase I kinase activity versus Mg^{2+} concentration with and without adenosine 3′,5′ cyclophosphate. Experimental conditions as in Fig. 3. Note that more experimental points were obtained at lower Mg^{2+} concentrations, which gives very high or infinite values for $1/v$ and could not be plotted.

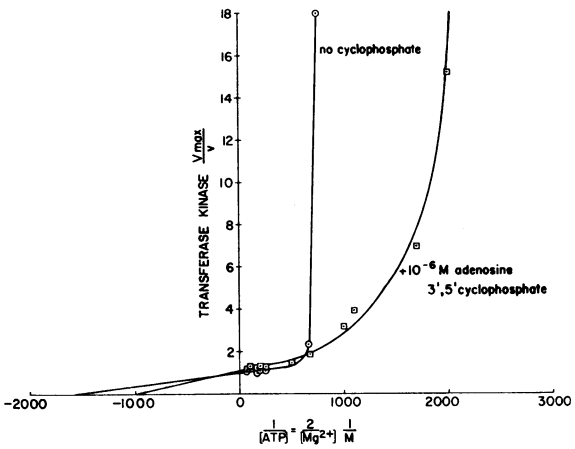

FIG. 5.—Double reciprocal plot of the relative velocity (V/v) of the transferase I kinase activity versus the Mg^{2+} concentration, which in this experiment is varied together with the ATP concentration, keeping the $[ATP]/[Mg^{2+}]$ ratio constant at 2. Experimental conditions otherwise as indicated in Fig. 1. More experimental points were obtained at low Mg^{2+} concentration, which had a very high or infinite value of V/v.

strate of transferase I kinase is an ATP-Mg complex. At low concentrations of the activator, i.e., Mg^{2+}, there is no activity because the dissociation constant of the enzyme-substrate complex is large [K_s, eq. (1)]. However, once an enzyme-activator complex is formed [eq. (2)], the substrate can be more effectively bound to the enzyme-activator complex [eq. (3)]. By constructing theoretical curves for the plot of V/v against $1/[Mg^{2+}]$ for different values of K_a[20] and comparing these to the experimental curves, it can be estimated that the dissociation constant of the enzyme-activator complex (K_a) in the absence of adenosine 3',5' cyclophosphate is of the order of $5 \times 10^{-2}\ M$. In the presence of the cyclic nucleotide, K_a can be determined to be approximately $1 \times 10^{-3}\ M$.

$$E + MgATP \overset{K_s}{\rightleftharpoons} E - MgATP \qquad K_s \text{ large number} \tag{1}$$

$$E + Mg^{2+} \overset{K_a}{\rightleftharpoons} E - Mg \text{ (activated enzyme)} \qquad \begin{matrix} K_a \text{ without cyclophosphate } 5 \times 10^{-2}\ M \\ K_a \text{ with cyclophosphate } 1 \times 10^{-3}\ M \end{matrix} \tag{2}$$

$$E - Mg + MgATP \overset{K_s'}{\rightleftharpoons} \begin{matrix} Mg \\ \diagup \\ E \\ \diagdown \\ ATPMg \end{matrix} \qquad \begin{matrix} K_s' \text{ without cyclophosphate } 2 \times 10^{-3}\ M \\ K_s' \text{ with cyclophosphate } 0.5 \times 10^{-3}\ M \end{matrix} \tag{3}$$

Thus, adenosine 3',5' cyclophosphate causes a small decrease (4-fold) in the dissociation constant of the activated enzyme-substrate complex [K_s', eq. (3)] but a major change (about 50-fold) in the dissociation constant of the enzyme-activator complex [K_a, eq. (2)]. It is of interest that Rosenberg has found similar nonlinear kinetics in studies of the activation of carnosinase by Mg^{2+}, which he has interpreted similarly.[22] Changeux[23] and Monod *et al.*[24] have discussed several enzymes in which the substrate is also an activator binding at an allosteric site different from the active site of the enzyme. These enzymes are activated by other metabolites as well. For example, when threonine deaminase is activated by threonine, an upward curved Lineweaver-Burk plot is obtained. If the enzyme is activated with norleu-

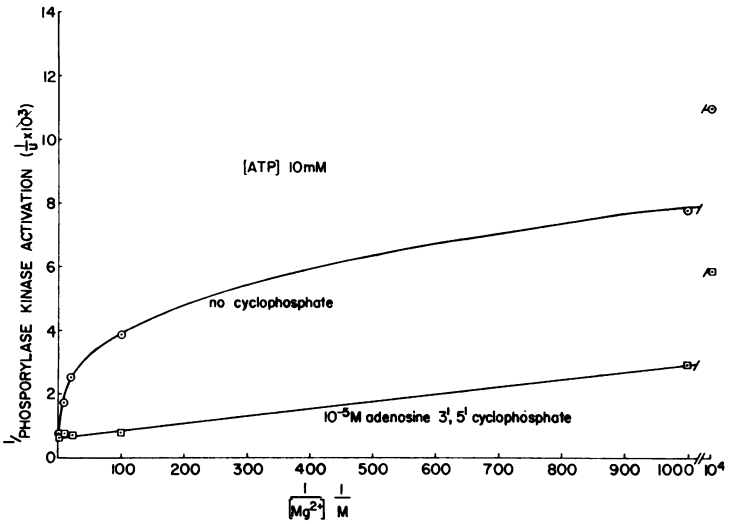

FIG. 6.—Replotted data of Krebs *et al.*[14] Double reciprocal plot of phosphorylase *b* kinase activation versus Mg^{2+} concentration at 10 mM ATP with and without adenosine 3′,5′ cyclophosphate.

cine, threonine apparently binds only to the active site and a straight line is obtained. However, in case of the transferase I kinase, both curves of the Lineweaver-Burk plot (Fig. 4) with and without adenosine 3′,5′ cyclophosphate are nonlinear. This indicates that even in the presence of the cyclic nucleotide, Mg^{2+} is still an activator but with an altered affinity constant.

Krebs *et al.*[14] studied the activation of phosphorylase *b* kinase varying the Mg^{2+} concentration in the presence and absence of adenosine 3′,5′ cyclophosphate. At low Mg^{2+} concentrations there was no phosphorylase *b* kinase activity in the absence of the cyclic nucleotide. Higher Mg^{2+} concentrations (up to 200 mM in the presence of 10 mM ATP) maximally stimulated even in the absence of cyclic nucleotide. We have taken the liberty of plotting the data of Krebs *et al.*[14] in a double reciprocal plot as shown in Figure 6. In the absence of cyclic nucleotide, a curved line which is convex upward is obtained. This is compatible with a mechanism involving two enzymes requiring the same cofactor, Mg^{2+},[20] one having a K_m for Mg^{2+} of the order of 10^2 higher than the K_m of the second. In the presence of adenosine 3′,5′ cyclophosphate, a straight line is obtained. This might indicate that now only one enzyme is operating, or that the two enzymes now have the same low K_m for Mg^{2+}.

The double reciprocal plot of phosphorylase *b* kinase activation versus ATP gives a similar kinetic picture to that which was seen in the case of the transferase I kinase, i.e., a straight line at low ATP concentration and inhibition at high ATP concentration. In both cases it is likely that the inhibition at high ATP is due to a removal of Mg^{2+}.

Mansour has studied the substrate velocity relationship of heart phosphofructokinase for fructose-6-phosphate.[25] In the absence of the cyclic nucleotide, a sigmoidal second-order curve was found. In the presence of the cyclic nucleotide

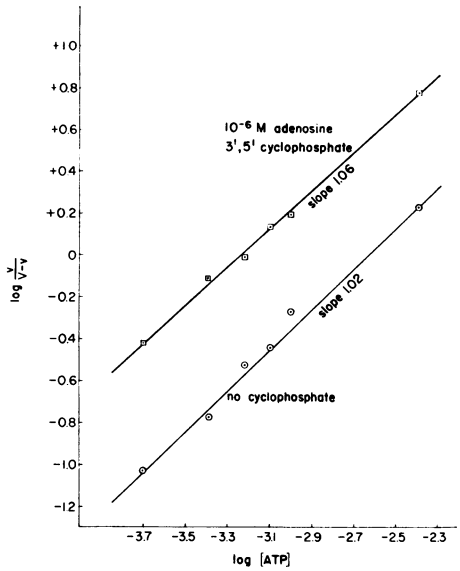

FIG. 7.—"Hill plot"[22] of the transferase I kinase as a function of log ATP (M) concentration. When the modified Michaelis-Menten equation $v = V/[(K_m/S_n) + 1]$, where n is the "degree of cooperation" between different binding sites of the enzyme for its substrate, is rearranged to log $v/V\text{-}v = n$ log [ATP] $-$ log K_m, the slope of the plot of log $v/V\text{-}v$ versus log ATP is equal to n. Experimental conditions as in Fig. 1.

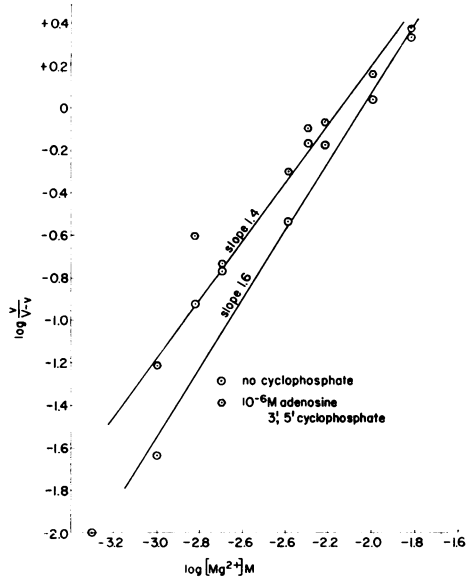

FIG. 8.—"Hill plot" of the transferase I kinase as a function of the log Mg^{2+} concentration. Although the points show a large scatter, it is clear that the slope of any line drawn through them is > 1. Experimental conditions as in Fig. 3.

(10^{-4} M), the more usual first-order hyperbolic curve was found. Thus, under the experimental conditions employed, the kinetics of the enzyme were markedly altered in the presence of the cyclic nucleotide. It seems likely that the effect observed by Mansour is of a different type than that observed in the present experiments with transferase I kinase and the experiments with phosphorylase b kinase for several reasons. The concentrations of the cyclic nucleotide required in the latter cases are about 10^{-3} to 10^{-4} M lower than in the former case (10^{-8} M as compared to 10^{-4} to 10^{-5} M). In addition, in the case of heart phosphofructokinase the cyclic nucleotide appears to act in a partially competitive manner with regard to the action of ATP which itself is inhibitory. Thus, the cyclic nucleotide reverses the inhibitory action of ATP. In the present experiments at all concentrations of ATP (even those which are inhibitory), the cyclic nucleotide promotes the action of ATP (see Fig. 1).

In order to get an impression of the "degree of cooperation" of different binding sites for Mg^{2+} on the transferase I kinase, the data were plotted according to Monod *et al.*[24] Figure 7 shows that the plot of the logarithm of (v) divided by ($V\text{-}v$) versus the logarithm of the ATP concentration yields lines with a slope of 1. Figure 8 shows that the same plot for the logarithm of the Mg^{2+} concentration gives a line with a slope of between 1 and 2.

At low Mg^{2+} concentrations adenosine 3′,5′ cyclophosphate controls the rate of the transferase I kinase reaction; in the absence of cyclic nucleotide there is no

measurable reaction. This effect is observed at a very low concentration of the cyclic nucleotide. We have previously reported that 6×10^{-8} M cyclic nucleotide gives half-maximal and 5×10^{-7} M maximal stimulation at an ATP concentration of 10 mM and a $MgCl_2$ concentration of 5 mM.

In conclusion, these experiments suggest that Mg^{2+} causes an allosteric activation of transferase I kinase and that adenosine 3′,5′ cyclophosphate increases the degree of affinity of the allosteric site of the enzyme for Mg^{2+}.

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