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Protein phosphorylation as a regulatory device

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Phosphorylation–dephosphorylation cascade systems represent a major mechanism of cellular regulation. A theoretical analysis of such systems (Stadtman & Chock 1977) revealed that they are endowed with extraordinary regulatory capacities; they may exhibit smooth, flexible responses to changes in single and multiple metabolite levels, signal amplification, and apparent positive cooperativity. To test the theories and equations involved in this analysis, an *in vitro* phosphorylation–dephosphorylation model system was developed. The system consists of a cyclic-AMP-dependent protein kinase and a phosphoprotein phosphatase, both isolated to near-homogeneity from bovine heart, and a nanopeptide that serves as the interconvertible substrate. Experiments with the model system confirm the predictions about the behaviour of a monocyclic cascade. They also reveal that when the concentration of enzyme–substrate complex is not negligible, cyclic cascades are potentially more sensitive to variations in effector concentrations and can achieve even greater signal amplification than previously predicted.

Introduction

Almost 40 years ago, it was observed (Cori & Green 1943; Cori & Cori 1945) that glycogen phosphorylase exists in two interconvertible forms, a relatively inactive form, phosphorylase *b*, and a more active form, phosphorylase *a*. Later studies demonstrated that the conversion of phosphorylase *b* to phosphorylase *a* involved an ATP-dependent phosphorylation of the enzyme by a highly specific protein kinase and that the conversion of phosphorylase *a* back to phosphorylase *b* was catalysed by a phosphoprotein phosphatase (for reviews see Krebs (1972) and Fischer *et al.* (1971)). Since then the cyclic phosphorylation and dephosphorylation of other enzymes (proteins) has been shown to be implicated in the regulation of diverse metabolic processes (Krebs & Beavo 1979; Stadtman & Chock 1978, 1979; Chock *et al.* 1980).

A theoretical analysis of interconvertible enzyme systems

The theoretical model

A scheme depicting the cyclic interconversion of an enzyme between phosphoryated and non-phosphorylated forms is shown in figure 1. The scheme consists of two opposing 'cascades' – so called because they involve the action of one enzyme upon another. In the forward cascade, the phosphorylation of the interconvertible enzyme, E, is catalysed by the active form of a protein kinase, PK_a. In the reverse cascade, the dephosphorylation of the phosphorylated interconvertible enzyme, E–P, is catalysed by the active form of a phosphoprotein phosphatase, PT_a. To obtain a better understanding of the characteristics of cyclic cascade systems, a theoretical analysis of the model depicted in figure 1 was carried out (Stadtman & Chock 1977). In that analysis, it was assumed that the phosphorylation cascade is triggered by the binding of an effector, e_1, to the inactive form of a protein kinase (PK_I), thereby converting it to an active

form, PK_a, which catalyses the phosphorylation of the interconvertible enzyme, E. Effector e_1 is therefore assumed to play a role similar to that of cyclic AMP in the activation of some protein kinases. In an analogous manner, the dephosphorylation cascade is triggered by the binding of effector e_2 to the inactive form of phosphoprotein phosphatase (PT_i), thereby converting it to

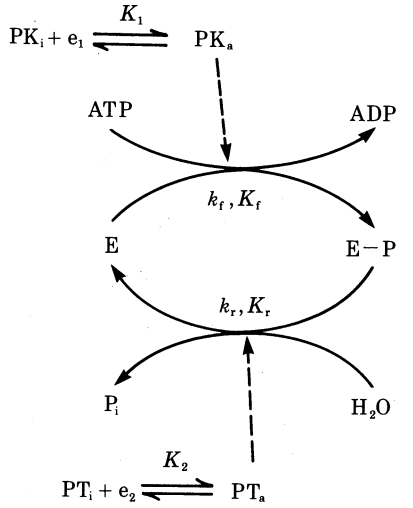

FIGURE 1. Schematic representation of a monocyclic cascade system. PK_i and PK_a represent, respectively, concentrations of the inactive and active forms of a protein kinase; PT_a and PT_i represent, respectively, concentrations of the active and inactive forms of a phosphoprotein phosphatase; K_1 and K_2 represent association constants for the $PK_a \cdot e_1$ and the $PT_a \cdot e_2$ complexes, respectively; k_f and k_r represent the specific rate constants for the phosphorylation and dephosphorylation steps, respectively; K_f and K_r represent the association constants for the $PK_a \cdot E$ and $PT_a \cdot E$–P complexes, respectively; E and E–P represent the unphosphorylated and phosphorylated forms of the interconvertible enzyme, respectively; e_1 and e_2 refer to the allosteric effectors.

the active form, PT_a, which catalyses the dephosphorylation of E–P. Obligatory coupling of the two cascades stems from the sharing of common substrates and products, and leads to the dynamic interconversion of E between phosphorylated and dephosphorylated forms. Moreover, with each complete cycle, one equivalent of ATP is converted to ADP and P_i.

The rate of the phosphorylation cascade is governed by the concentrations of PK, e_1 and E, by the specific rate constant k_f, and by the equilibrium constants K_1 and K_f (see Stadtman & Chock (1977) for details). The rate of the dephosphorylation of E–P is governed by a similar set of parameters, specifically [PT], $[e_2]$, [E–P], k_r, K_2 and K_r. It follows that for a given metabolic condition, a steady state will be attained in which the rate of phosphorylation of E is equal to the rate of dephosphorylation of E–P. Furthermore, a change in the value of any one of the ten parameters that govern the rates of the phosphorylation–dephosphorylation reactions will lead automatically to a shift in the steady state and, concomitantly, to a shift in the distribution of phosphorylated and non-phosphorylated enzyme species. The theoretical analysis of such a model shows that the fraction of phosphorylated enzyme that will exist in the steady state is determined by

$$\frac{[\text{E–P}]}{[\text{E}]} = \left\{R\left(\frac{[e_2]}{[e_1]}\right)\left(\frac{1+K_1[e_1]}{1+K_2[e_2]}\right)+1\right\}^{-1}, \tag{1}$$

where R is the product of four different parameter ratios:

$$R = \left(\frac{k_r}{k_f}\right)\left(\frac{K_2}{K_1}\right)\left(\frac{K_r}{K_f}\right)\left(\frac{[\text{PT}]}{[\text{PK}]}\right) \tag{2}$$

In these formulations, [E], [PK] and [PT] represent the total concentrations of the interconvertible enzyme, protein kinase and phosphoprotein phosphatase, respectively, and [E–P] represents the steady-state concentration of phosphorylated interconvertible enzyme. Other parameters are as indicated in figure 1. The steady state is also dependent upon the concentration of ATP, but this parameter was ignored in the theoretical analysis because the intracellular concentration of ATP is maintained at a nearly constant level, several orders of magnitude greater than the concentrations of the cascade enzymes.

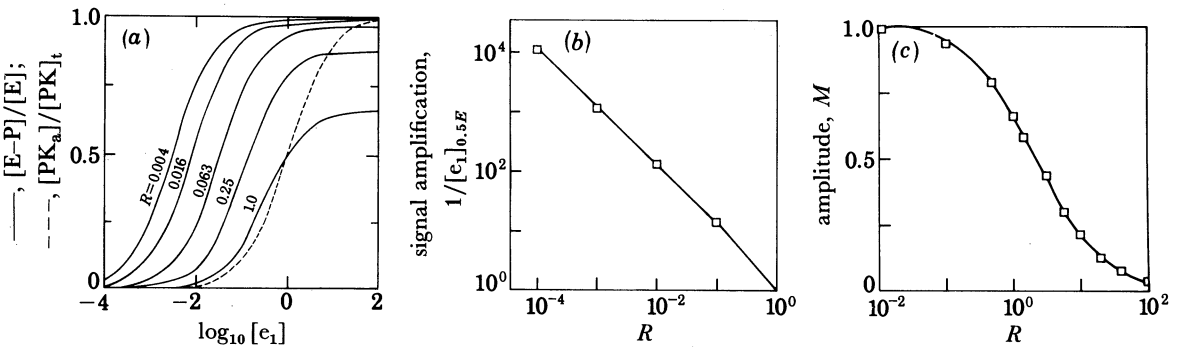

FIGURE 2. Computer simulated curves obtained with equation (1) to illustrate the following: (*a*) that the relation between fractional phosphorylation of an interconvertible enzyme and effector concentration, $[e_1]$, varies as a function of R, the product of all parameter ratios shown in equation (2); (*b*) the relationship between R and the signal amplification (see text for definition); (*c*) the changes in amplitude as a function of R. In (*a*) the solid curves refer to $[E–P]/[E]_t$ and the broken line refers to $[PK_a]/[PK]_t$. K_1 was assumed to be 1.0 concentration unit.

Properties of cyclic cascade systems

Some remarkable properties of cyclic cascades are demonstrated by the computer-simulated curves in figure 2 showing how, according to (1), the steady-state level of phosphorylated enzyme varies as a function of the concentration of e_1. The broken line in figure 2 shows how the fraction of PK_a varies as a function of concentration of e_1 with the assumption that $K_1 = 1$ and that the binding of e_1 to PK_i follows Michaelis–Menten behaviour. Thus when e_1 is present at a concentration of unity, half of the PK is in the active form PK_a. The other curves in figure 2 show how the phosphorylation of E varies with concentration of e_1 when the binding of e_1 to PK is linked to the cyclic cascade system through the PK_a-catalysed phosphorylation of E. Note that when $R = 1.0$ (i.e. when all parameter ratios in (2) equal unity), the concentration of e_1 required to obtain 50 % phosphorylation of E is also equal to 1.0; moreover, under this condition, a maximum of only 67 % of E can be phosphorylated when PK is completely saturated with e_1. In other words, the amplitude of the phosphorylation state is only 67 % of the maximum value. The other curves in figure 2 show that as R is decreased from 1.0 to 0.004, there is a progressive increase in the amplitude of phosphorylation of E and a progressive decrease in the concentration of e_1 required to achieve a steady state in which 50 % of E is phosphorylated.

Note also that a concentration of e_1 (0.01) that causes less than 2 % activation of the protein kinase can, depending on the value of R, result in almost complete phosphorylation of the interconvertible enzyme. In other words, cyclic cascades are endowed with an enormous capacity for 'signal amplification', which for quantitative comparisons has been defined as the ratio of the concentration of e_1 required to achieve 50 % activation of PK to that required to produce

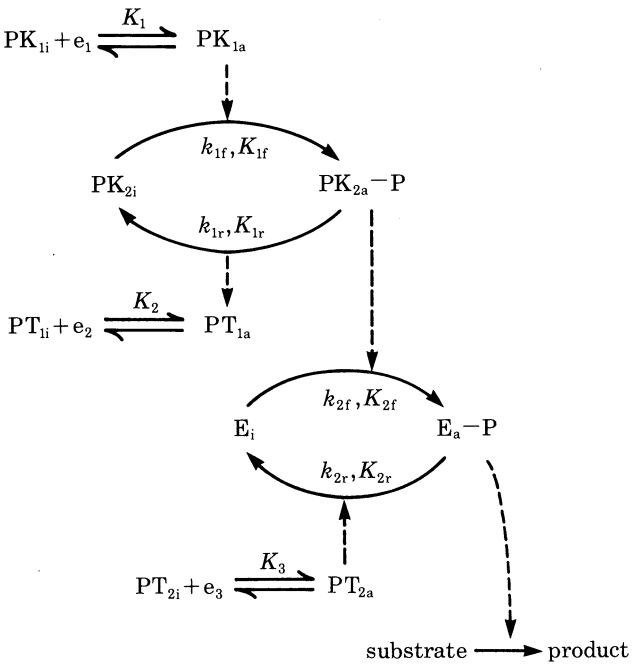

FIGURE 3. Schematic representation of a bicyclic cascade system. The subscripts 1 and 2 refer to constants in the first and second cycles, respectively; otherwise, notations are as described in figure 1.

50 % phosphorylation of the interconvertible enzyme (Stadtman & Chock 1978). It is so defined because the concentration of allosteric effector, e_1, is the metabolic signal that triggers the cyclic cascade. The relation between the parameter ratio, R, and the signal amplification and the amplitude for a monocyclic cascade is illustrated in figure 1*b* and 1*c* respectively. It should be emphasized that because R is the product of four parameter ratios (see (2)), relatively small changes in each parameter will result in very large changes in R. For example, R can change from 1.0 to 0.004 with only a twofold increase in each parameter that governs the phosphorylation of E, together with a 50 % decrease in each parameter governing the dephosphorylation of E–P.

Flexibility of regulatory patterns

With the assumption that the phosphorylation of E is always triggered by the binding of e_1 to PK_1, monocyclic cascades may exhibit very different patterns of regulation, depending upon whether e_1, in addition to its effect on PK, can also affect the activity of PT, and also whether e_1 and e_2 are inhibitors or activators of PT. Four possible ways in which e_1 and e_2 can react with PT are as follows: case I (illustrated in figure 1), $PT_1 + e_2 \rightarrow PT_a$; case II, $PT_a + e_2 \rightarrow PT_1$; case III, $PT_a + e_1 \rightarrow PT_1$; case IV, $PT_1 + e_1 \rightarrow PT_a$. A theoretical analysis (Stadtman & Chock 1977) shows that the response of the phosphorylation of E to increasing concentrations of e_1 is different in each of these cases with respect to amplitude, signal amplification and sensitivity (discussed below). Case III is of special interest because it generates a sigmoidal response of phosphorylation of E to increasing concentrations of e_1. This apparent cooperativity results from the fact that the same effector both activates the phosphorylation of E and inhibits the dephosphorylation of E–P. Because E, PK and PT may each react with more than one positive or negative allosteric effector, it is clear that by using simultaneously any combination of two or more of the four basic patterns described above, monocyclic cascades can

elicit a vast number of regulatory responses. In fact, three of the four cases described above are used in the regulation of mammalian pyruvate dehydrogenase activity (for review see Stadtman & Chock 1978).

Multicyclic cascades

As shown in figure 3, when the interconvertible enzyme in one cycle is a protein kinase that catalyses the phosphorylation of an interconvertible enzyme in a second cycle, the two cycles become coupled. Consequently the steady-state level of phosphorylated enzyme in the second cycle is a function of all parameters in both cycles; similarly, if the phosphorylated form of the interconvertible enzyme in the second cycle can catalyse the phosphorylation of a third interconvertible enzyme, then phosphorylation of the latter is a function of the parameters that govern all three cycles; and so on. Thus the addition of each new cycle to a cascade increases by eight (or more) the number of parameters that govern the activity of the interconvertible enzyme in the last cycle. As a consequence the potential for signal amplification increases exponentially as the number of cycles in the cascade is increased (Stadtman & Chock 1977). In addition, multicyclic cascades are responsive to many more allosteric effectors and therefore possess much greater regulatory flexibility than monocyclic cascades. Finally, because there are more steps in a multicyclic cascade than in a monocyclic cascade, they are potentially more sensitive in their response to increasing concentrations of effectors that can act at more than one step in the cascade (Chock & Stadtman 1977).

Rate amplification

In addition to the above properties, a theoretical analysis shows that the phosphorylation of the interconvertible enzyme in the last cycle of a multicyclic cascade proceeds with a lag followed by a burst in the rate of phosphorylation, and that the burst becomes steeper as the number of cycles in the cascade increases (Chock & Stadtman 1979; Stadtman & Chock 1979). The rapid increase in the rate is due to the fact that the rate of phosphorylation is a multiplicative function of the rate constants of all the forward steps in the cascade. Thus multicyclic cascades function as rate amplifiers. They are capable of eliciting a large change in the fractional phosphorylation of the interconvertible enzyme in the millisecond time range (Chock & Stadtman 1979).

Validity of the theoretical considerations

Most of the unique features of cyclic cascades have been confirmed in principle through detailed studies of the monocyclic cascade that regulates the phosphorylation of mammalian pyruvate dehydrogenase (Reed *et al.* 1980; Chock *et al.* 1978), and of the bicyclic cascade that regulates the adenylylation of glutamine synthetase from *Escherichia coli* (Rhee *et al.* 1978). However, both of these systems are so complex that a thorough quantitative analysis of them is fraught with difficulties. We therefore developed a simple *in vitro* model system that could be used to investigate the validity of the theoretical predictions. In this model, which is illustrated in figure 4, the nanopeptide Leu-Arg-Arg-Ala-Ser-Val-Ala-Gln-Leu was used as the interconvertible substrate (S) because it possesses only a single serine residue that can be phosphorylated and thus avoids complications that would arise if multiple phosphorylations took place. The converter enzymes, highly purified cyclic-AMP-dependent protein kinase (R_2C_2, type II) and phosphoprotein phosphatase (M_r = 38000) were prepared from bovine heart muscle.

The relation between the steady-state level of phosphopeptide, S–P, and the activities of the

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kinase and phosphatase is defined by an equation significantly different from (1). This is because the derivation of (1) was simplified by assuming that the concentrations of the complexes between the converter enzymes and the interconvertible substrate were negligibly small compared with the total concentrations of the converter enzymes. This assumption is reasonable for some interconvertible enzyme systems (such as the glutamine synthetase cascade (Rhee *et al.* 1978)), but it is not valid for the experimental model (figure 4) because the affinity of the protein kinase for the nanopeptide substrate is extremely high (K_m = 0.26 μM). To account for the fact that a significant fraction of the protein kinase is associated with the interconvertible peptide, a considerably more complicated equation is required to describe the

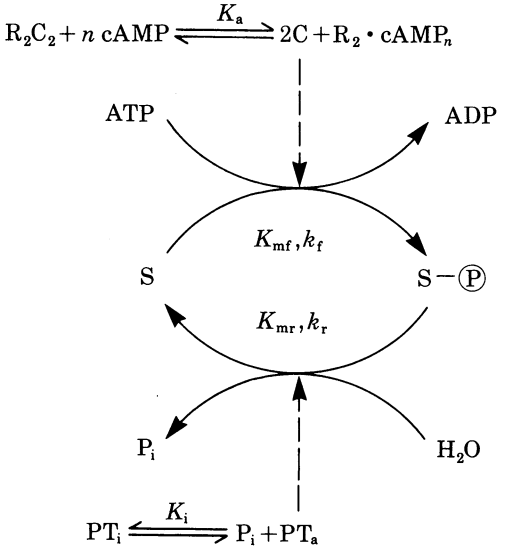

FIGURE 4. Model monocyclic phosphorylation–dephosphorylation cascade system. Cyclic-AMP-dependent protein kinase (R_2C_2), which is dissociated by cyclic AMP (cAMP) (n = 2 to 4) into a regulatory dimer (R_2) and two catalytic subunits (C), catalyses the phosphorylation of an interconvertible nanopeptide (S). The dephosphorylation of the phosphonanopeptide (S–P) is catalysed by an active phosphoprotein phosphatase (PT_a) that can be inhibited competitively by millimolar concentrations of P_i. The molar rate constants, k_f and k_r, the K_m values, K_{mf} and K_{mr}, and the dissociation constants for the enzyme–substrate complexes (i.e. S·C and S–P·PT_a, not shown) pertain to the forward and reverse cascades as shown. K_a and K_i are the concentrations of cyclic AMP and P_i that effect 50% activation and inhibition of the kinase and phosphatase, respectively.

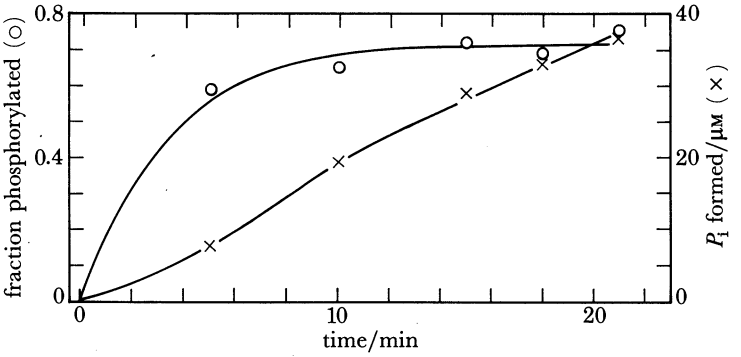

FIGURE 5. The attainment of a steady state in a cyclic cascade. The incorporation of phosphate into 10 μM nanopeptide (circles) and the release of P_i (×) were measured simultaneously, at the times indicated, in the standard cyclic cascade assay (25 mM MOPS, pH 7.0, 5 mM $MgCl_2$, 0.5 mM [γ-^{32}P]ATP, 2 mM DTT, 0.3 mg ml^{-1} BSA, 10% glycerol by volume) containing 0.5 μM cyclic AMP, 4.4 nM kinase and 1.3 μM phosphatase.

experimental model. Details of the mathematical derivation will be presented elsewhere. For the present discussion, it is only necessary to emphasize that all of the parameters in figure 4 have been experimentally determined by kinetic studies.

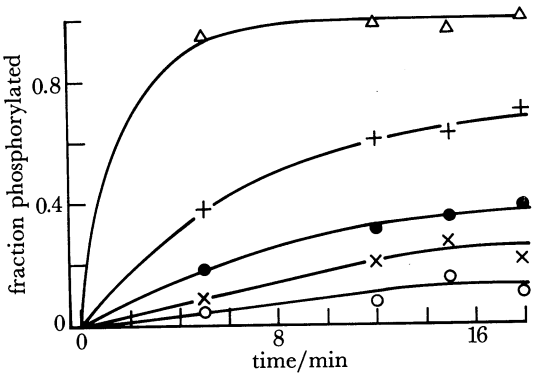

FIGURE 6. The dependence of steady-state level of phosphorylation on the concentration of cyclic AMP. The incorporation of phosphate into 10 μM nanopeptide was measured in the standard cascade assay (see legend to figure 5) containing 4.4 nM kinase and 0.44 μM phosphatase, and the following concentrations of cyclic AMP: none (○), 0.024 μM (×), 0.07 μM (●), 0.19 μM (+), 1.0 μM (△).

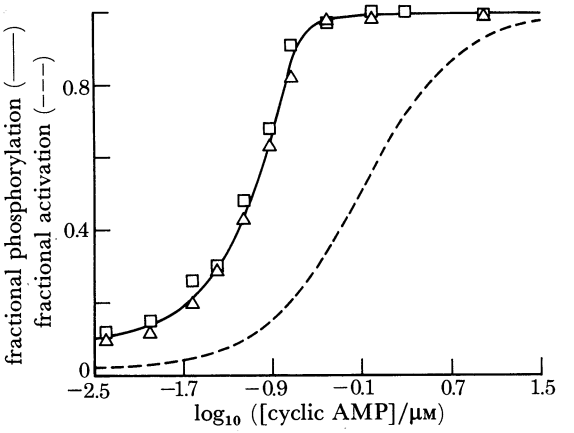

FIGURE 7. Steady-state fractional phosphorylation at various concentrations of cyclic AMP. The incorporation of phosphate into 10 μM nanopeptide was measured at steady state (16 min assay time; see figure 6) in the standard cascade assay containing 4.4 nM kinase, 0.44 μM phosphatase, cyclic AMP at the concentrations indicated (squares and triangles represent duplicate experiments). A steady-state equation that delineates the kinetics of the cyclic cascade was fitted to the data by a computer-assisted analysis. The values for the kinetic variables obtained for the best fit to the data (solid curve) will be given elsewhere. The broken line is a computer simulation of the fractional activation of cyclic-AMP-dependent protein kinase expected for the same enzyme concentration (4.4 nM) as that employed in the cyclic cascade; K_a was taken to be 0.8 μM.

The theoretical cyclic cascade model predicts that when all components of the cascade are present, a steady state will be established in which the rates of peptide phosphorylation and dephosphorylation are equal, and thus the fraction of phosphorylated peptide assumes a constant value. These predictions are confirmed by the data in figure 5, showing that the fraction of phosphorylated peptide increases with time and assumes a constant value of 0.7 under one set of conditions. It is apparent that this represents a dynamic state in which the rates of phosphorylation and dephosphorylation are equal because ATP decomposition, as measured by the accumulation of P_i, continues at a constant rate even after the concentration

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of phosphorylated peptide becomes constant. Because each complete phosphorylation–dephosphorylation cycle results in the conversion of one equivalent of ATP to ADP and P_i, the rate of generation of P_i is a measure of the rate of interconversion of the nanopeptide between phosphorylated and non-phosphorylated states. Note that the decomposition of ATP is neither a wasteful nor a futile process: it provides the energy that is needed to sustain steady-state levels of phosphorylated enzymes that are very different from those that would exist at thermodynamic equilibrium. The decomposition of ATP is in fact the price the cell must pay for an exquisite mechanism of cellular regulation.

The theoretical analysis predicts that the steady-state level of S–P is determined in part by the concentration of the effector cyclic AMP. This prediction is verified by the data in figure 6, showing that as the concentration of cyclic AMP is increased, the steady-state level of phosphorylated peptide also increases. The relation between the steady-state level of S–P and the concentration of cyclic AMP is illustrated in figure 7. The solid curve is a computer simulation curve obtained when the experimentally determined values of the various kinetic constants are incorporated in the theoretical equation that describes the model shown in figure 4. Excellent agreement between this curve and the experimentally determined levels of S–P as a function of the cyclic AMP concentration (figure 7, open symbols) attests to the validity of the model.

The results illustrate two other important features of the cyclic cascade: the capacity for signal amplification and the capacity for generating a cooperative response, i.e. sensitivity, to increasing concentrations of effector. A comparison of the two curves in figure 7 shows that the concentration of cyclic AMP required to produce 50 % phosphorylation of the peptide (solid line) is one-tenth of that required to obtain 50 % activation of the protein kinase (broken line). The signal amplification is therefore 10. Note also that the slope of the mid-portion of the fractional phosphorylation curve in figure 7 is considerably steeper than that of the PK activation curve. This reflects the fact that there is a sigmoidal response of peptide phosphorylation to increasing concentrations of cyclic AMP. Therefore, within the range of cyclic AMP concentrations around the midpoints of the curves, the phosphorylation of the peptide is more *sensitive* to increasing concentrations of cyclic AMP than is the activation of the kinase. As already noted, such apparent cooperativity is characteristic of cyclic cascades in which the same effector both activates the kinase and inhibits the phosphatase. However, the apparent cooperativity observed in studies of the experimental model derives from the fact that the catalytic subunit, C, of the protein kinase forms a very tight complex with the nanopeptide. The concentration of free C is therefore not large compared with the total concentration of C. In an independent study, Goldbeter & Koshland (1981) noted also that when a converter enzyme is saturated by its interconvertible enzyme substrate, the cyclic cascade can exhibit a sigmoidal response to effector concentration, termed 'zero-order ultrasensitivity'.

Flexibility

As emphasized earlier, cyclic cascades are extremely flexible in their regulatory behaviour because both the kinase and the phosphatase can be modulated simultaneously by changes in the concentrations of positive and negative effectors. This flexibility is demonstrated in the experimental model through studies in which the concentration of cyclic AMP, a positive effector of PK, and the concentration of P_i, a negative effector of PT, were both varied. The family of curves in figure 8 illustrates the effects of increasing cyclic AMP concentration on the fractional phosphorylation of S at various concentrations of P_i. It can be seen that as the

concentration of P_i is increased from 0 to 8 mM, there is a progressive decrease in the concentration of cyclic AMP required to achieve a given degree of peptide phosphorylation; i.e. the signal amplification increases. Although agreement between the experimental data (symbols) and the theoretical curves (solid lines) is not perfect (figure 8*a*), the lack of agreement may be

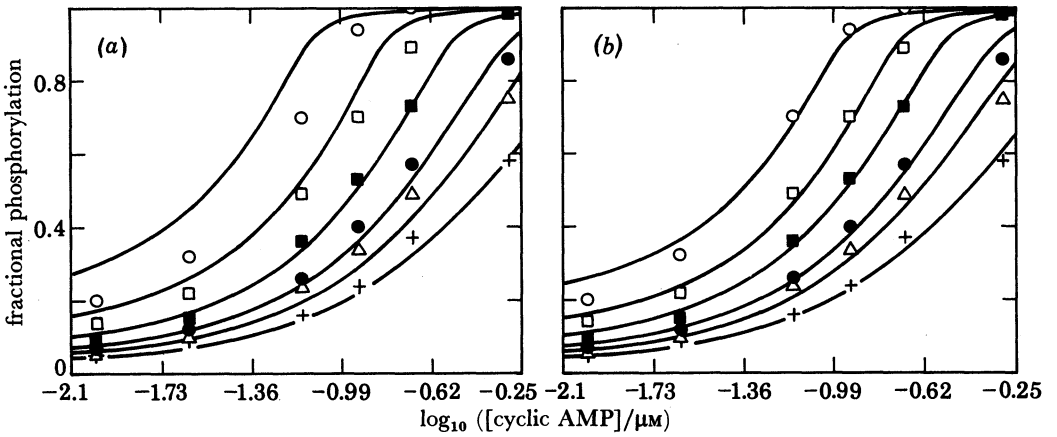

FIGURE 8. Steady-state fractional phosphorylation at varying concentrations of cyclic AMP and P_i. The incorporation of phosphate into 10 μM nanopeptide was measured at steady state (18 min assay time) in the standard cascade assay containing 4.4 nM kinase, 1.3 μM phosphatase, the concentrations of cyclic AMP indicated, and the following concentrations of phosphate: 0 mM (+), 0.5 mM (△), 1.0 mM (●), 2.0 mM (■), 4.0 mM (□), 8.0 mM (○). Each solid curve is a computer simulation of the steady-state fractional phosphorylation for one concentration of P_i, based upon an experimentally determined K_i of 1.5 mM. In (*a*), all kinetic constants employed in the analysis are the same as those employed in figure 7, in which K_a was 0.8 μM; (*b*) differs from (*a*) only in that the curves were adjusted to the data by allowing K_a to vary between 0.74 and 1.19 μM.

attributable to experimental error or possibly to small effects of concentration of P_i on the affinity of cyclic AMP for the protein kinase. As shown in figure 8*b*, a reasonably good fit of the data points to the theoretical curves is achieved by changing only slightly (from 0.74 to 1.19 μM) the assumed K_a values for cyclic AMP.

DISCUSSION

The phosphorylation of some interconvertible enzymes leads to their activation, whereas phosphorylation of other enzymes leads to inactivation. It has therefore been suggested that interconvertible enzymes are 'metabolic switches' through which metabolic pathways can be turned on or off in response to metabolic stimuli. However, this concept is unrealistic because it implies that the activities of the protein kinases and the phosphoprotein phosphatases vary reciprocally in an all-or-none manner, or, more accurately, that the concentrations of effectors that regulate their activities vary reciprocally in an all-or-none manner. This view is not supported by studies with either mammalian pyruvate dehydrogenase or glutamine synthetase from *E. coli*, for which the steady-state levels of covalent modification vary over wide ranges depending upon the metabolic state of the cell. Hence the interconversion of enzymes between phosphorylated and non-phosphorylated states is probably a dynamic process by means of which the fraction of phosphorylated enzyme, and hence the specific enzyme activity, can be varied gradually and continuously over a wide range, commensurate with metabolic demands.

Considering the fact that the activities of protein kinases and phosphoprotein phosphatases

might be affected by more than one effector, it is clear that cyclic cascades are uniquely designed for the fine regulation of key enzymes. They are in effect biological integration systems. By means of multisite interactions between the converter enzymes and allosteric effectors, they can sense fluctuations in the concentrations of multiple metabolites. Consequently, changes in the concentrations of any one or more of the allosteric effectors lead automatically to shifts in the steady-state levels of phosphorylation and therefore to automatic adjustments of the interconvertible enzyme activity.

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