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AUTOPHOSPHORYLATION OF CARDIAC 3′,5′-CYCLIC AMP-STIMULATED PROTEIN KINASE

KINETIC EVIDENCE FOR THE REGULATORY SUBUNIT DIRECTLY ACTING AT THE ACTIVE SITE IN THE R_2C_2 COMPLEX

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Summary

The mechanism of the autophosphorylation reaction of bovine heart cyclic AMP-dependent protein kinase (ATP : protein phosphotranferase, EC 2.7.1.31) has been further examined using a kinetic approach. The reaction is first order in both ATP and protein kinase when both are present at comparable concentrations. Dilution has no effect on the fraction of regulatory subunit phosphorylated over a given interval of time, and this finding is in accord with the autophosphorylation proceeding via an intramolecular (or, more appropriately in this case, by an intracomplex) reaction. The possibility of regulatory subunit phosphorylation by uncomplexed catalytic subunit or another R_2C_2 complex (the protein kinase complex of two catalytic subunits and the regulatory dimer) was clearly eliminated. These results are compatible with a subunit geometry permitting the regulatory subunit to bind at the protein substrate region of the kinase's active site and to undergo subsequent phosphorylation.

Work in Rosen's laboratory [1,2] has revealed that the purified cyclic adenosine 3′ : 5′-monophosphate-dependent protein kinase (ATP : protein phosphotransferase, EC 2.7.1.37) of bovine cardiac muscle catalyzes the reversible incorporation of up to 2 mol of ^{32}P from [γ-^{32}P]ATP into seryl residues of the cyclic AMP binding protein (regulatory subunit, R) **. Phosphorylation of the enzyme facilitates its dissociation by cyclic AMP, but it is not thought to affect cyclic AMP binding [1]. Apparently, at early stages of the purification the protein kinase is highly phosphorylated but loses the seryl-bound phosphates dur-

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** In this report we use R_2C_2 as an abbreviation for the protein kinase complex of two catalytic subunits and the regulatory dimer.

ing later steps in that procedure [2]; and it is known that the broadly specific cardiac phosphoprotein phosphatase can also remove the bound phosphate [1,2]. The protein kinases of porcine heart [3] and bovine brain [4] also appear to mediate self-phosphorylating reactions, and the occurrence of autophosphorylation may thus provide for a second level of control which is not clearly understood.

The mechanism of this auto-activating process has been of interest to us. In particular, does the regulatory subunit play a role akin to soybean trypsin inhibitor by forming a highly affine, inactive complex at the protein substrate site? Does autophosphorylation then serve to convert the regulatory subunit to a phosphoprotein resembling the reaction product and possibly thereby facilitating the cyclic AMP-dependent dissociation? Or, does the regulatory subunit act at a specific allosteric site which is topologically distinct from the protein substrate adsorption pocket of the active site? Or, lastly, does the process occur by the action of the free catalytic subunit (C) on yet another inactive protein kinase dephospho-R_2C_2 complex?

It was felt that the first step in the distinction between these possibilities should be an examination of the R_2C_2 concentration dependence of ^{32}P incorporation from γ-labelled ATP into the regulatory subunits. If an intermolecular process between C and R_2C_2 (or two R_2C_2 complexes) is required, then the rate of incorporation should be sensitive to dilution of the enzyme. However, for intramolecular phosphorylation the rate of autophosphorylation should be independent of enzyme concentration provided that the R_2C_2 complex has not dissociated on dilution. We have examined the stability of the R_2C_2 complex over the concentration range of 1—1000 μg/ml in the presence of varying histone or ATP levels by using both the loss of cyclic AMP dependence and increase in the specific activity in the absence of cyclic AMP as indicators of dissociation. No significant dissociation was observed. Working within this range, it has been possible to show that the rate of autophosphorylation is R_2C_2 concentration-independent, suggesting an intramolecular process. Consonant with this observation is the finding that changes in the ATP affect only that fraction of R_2C_2 which is phosphorylated, but not the R_2C_2 concentration independence of the rate of autophosphorylation.

Materials and Methods

Beef hearts were obtained promptly from the killed animals and transported to the laboratory in an ice chest. Ventricular tissue was stored at —80° C until used. Protein kinase was isolated by the procedure described by Rubin, Erlichman and Rosen [5], except that EDTA in the initial extraction buffer was 0.1 mM, and Bio-Gel A 0.5 m was used in gel filtration in place of Bio-Gel P300. Purity was monitored by enzyme specific activity assays and disc gel electrophoresis [5]. The final preparation was 7-fold stimulated by 1 μM cyclic AMP and comprised about 90% of the protein as estimated by the intensity of staining of the disc gels. The preparation was free of detectable phosphoprotein phosphatase, ATPase, and adenylate kinase.

[γ-^{32}P]ATP was prepared by the method of Glynn and Chappell [6] using [^{32}P] orthophosphoric acid obtained from ICN-Nuclear. [U-^{14}C]ATP was from

Amersham Searle. Nucleotide preparations were standardized spectrophotometrically and checked for homogeneity by DEAE-paper chromatography.

Protein concentrations were determined by the method of Lowry et al. [7]. Calculations of protein kinase concentration were based on a molecular weight of 174 000 [2]. Phosphoprotein phosphatase was prepared according to the procedure of Nakai and Thomas [8] by Ms. Hillary D. White of this laboratory.

Results

In this study, two models were considered for autophosphorylation. The first is an intermolecular process involving tetramer-tetramer or catalytic subunit-tetramer interactions. This is shown in Scheme I:

$$RC + ATP \rightleftharpoons RC \cdot ATP$$

$$RC \cdot ATP + RC \rightleftharpoons RC \cdot ATP \cdot RC \rightarrow RC + ADP + (R\text{-}P)C \qquad \text{Scheme I}$$

Here, RC, R-P, and C represent the regulatory-catalytic subunit complex, the phosphorylated regulatory subunit, and the free, active catalytic unit, respectively. Since no evidence of catalytic subunit interaction was observed in this study, the above scheme and those which follow relate to the catalytic subunit on each half of the R_2C_2 tetramer and assume that the C subunits of this tetrameric structure are independent of one another. The rate equation for this mechanism predicts a second order dependence on enzyme and a complex order dependence * on ATP concentration. Under conditions where the initial levels of enzyme and ATP are similar or within one or two orders of magnitude of one another (conditions used in this study to compensate for the great rapidity of autophosphorylation at high ATP and the large errors in assay at low enzyme), the velocity expression was treated by the use of initial velocity meausrements. With such restrictions, the initial reaction velocity should be second order with respect to $[R \cdot C]_{total}$ and first order with respect to [ATP]. This requires a parabolic curve in a plot of v_0 vs. $[RC]_{total}$ or a straight line with slope equal to 2 in plots of $\log v_0$ vs. $\log [RC]_{total}$, commonly known as Van 't Hoff plots [9]. The second model considered was an intramolecular process, shown in Scheme II:

$$RC + ATP \rightleftharpoons RC \cdot ATP$$

$$RC \cdot ATP \rightarrow (R\text{-}P)C + ADP \qquad \text{Scheme II}$$

The velocity expression for this latter process predicts a first order dependence on RC and again a complex order ATP dependence. Under the initial rate conditions, this simplifies to an expression that is first order in either RC or ATP concentration. This would result in a linear relationship in plots of v_0 vs. $[RC]_{total}$ and straight lines with slope equal to 1 in the so-called Van 't Hoff plots.

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* By this we mean that the observed reaction order is dependent upon the fractional saturation of the ATP site of the catalytic subunit, just as the reaction order of any enzyme displaying Michaelis-Menten kinetics.

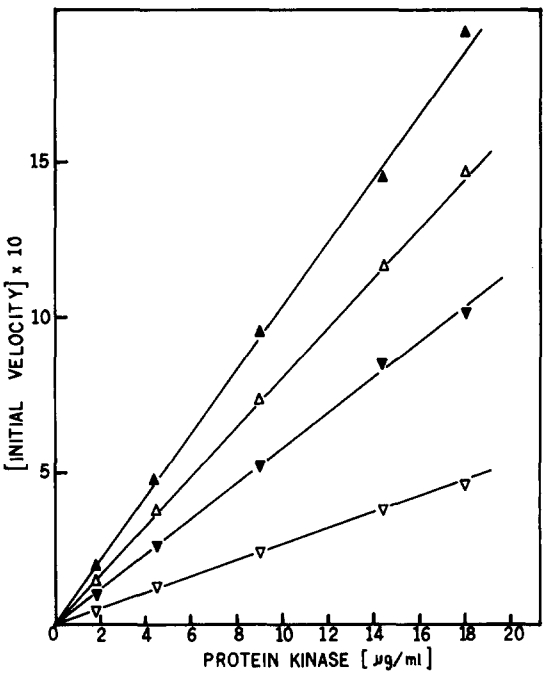

Fig. 1. Plot of the initial velocity (expressed as nM phosphorylated enzyme per min) vs. the initial concentration of protein kinase (μg/ml) at several levels of ATP. Assay mixture contained 50 mM potassium phosphate (pH 7.0), 7.5 mM $MgCl_2$, 5 mM 2-mercaptoethanol a variable amount of protein kinase as indicated, and [γ-^{32}P]ATP (13 500 cpm/pmol) at 0.2 mM (▲), 0.15 mM (△), 0.10 mM (▼), and 0.05 mM (▽).

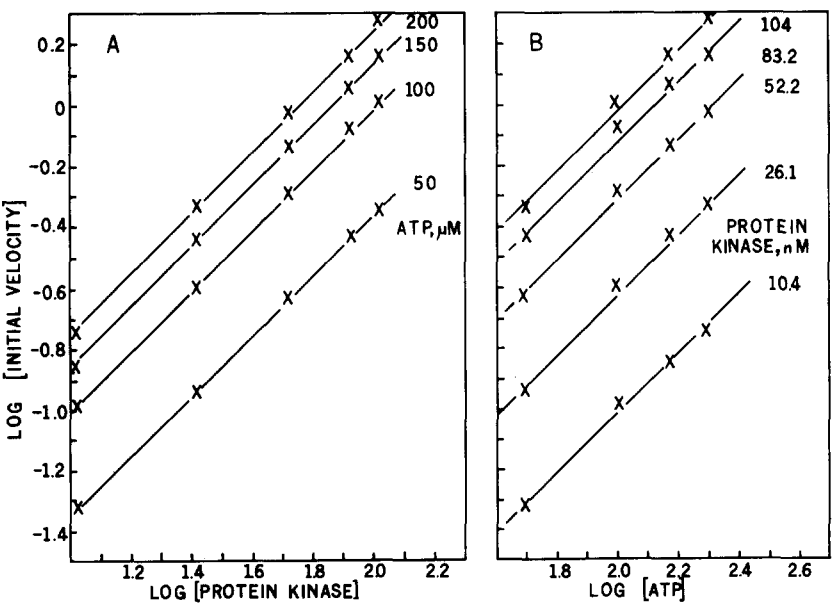

Fig. 2. A, Van 't Hoff plot of the data of Fig. 1 with respect to protein kinase concentration; B, Van 't Hoff plot of the data of Fig. 1 with respect to ATP concentration.

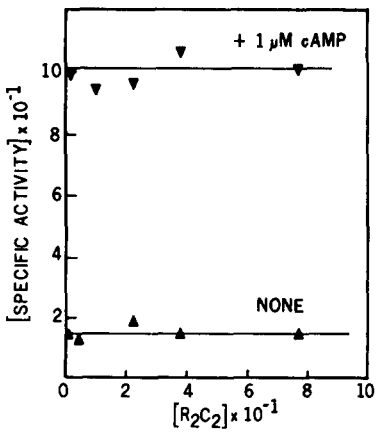

Fig. 3. Plot of the specific activity of protein kinase (cpm ^{32}P incorporated into histone/min per μg kinase) as a function of protein kinase concentration (μg/ml) in the standard histone assay. The 3',5'-cyclic AMP concentration was none (▲) and 1.0 μM (▼).

The results of initial velocity measurements on the autophosphorylation of bovine heart protein kinase are presented in Figs. 1 and 2. Fig. 1 represents plots of v_0 vs. $[RC]_{total}$ at varying concentrations of ATP. At all levels of ATP the initial velocity is a linear function of $[RC]_{total}$. Figs. 2A and B depict Van 't Hoff plots with respect to $[RC]_{total}$ and $[ATP]_{total}$, respectively, at different levels of co-reactant. In each case the slope of the Van 't Hoff plot is 1. These results are clearly in accord with the intramolecular model for autophosphorylation and stand against significant kinetic contributions from processes of the intermolecular type.

A complication might occur if the enzyme dissociates upon dilution to give free catalytic and regulatory subunits. For example, if the free catalytic subunit were intrinsically more active than the RC complex, such dissociation might fortuitously mask the second order nature of the intermolecular kinetics with respect to $[RC]_{total}$. Over the range of concentrations studied (Fig. 3), both the specific activity of the protein kinase in the absence of cyclic AMP (which would increase as dissociation occurred) and the stimulation propensity of cyclic AMP (which would decrease with dissociation) were found to remain constant, suggesting that dissociation is not appreciable. This stability of the bovine heart tetramer toward dissociation is in contrast to the brain enzyme which is apparently fairly easily dissociable [10].

The extent of incorporation of radiophosphorus by autophosphorylation was found not to exceed about 40% of the regulatory subunits. This is at variance with the data of Erlichman and Rosen [1,2] whose values have at times approached 100%, but our value is comparable to values of 63% and 46% reported by Maeno et al. [4]. The possibility that the enzyme may have been isolated in a predominantly phosphorylated form was examined further by adding ADP to the autophosphorylation mix to promote the equilibrium exchange of ^{32}P into the phosphoprotein and by preincubating the enzyme with phosphoprotein phosphatase. In the absence of ADP virtually no exchange was observed and the maximal level of autophosphorylation was found to remain

constant over long periods, indicating an absence of ATPase and phosphoprotein phosphatase activities. The absence of ADP prevented any significant reverse reaction from phospho-R under conditions for the determination of initial reaction velocities. Pretreatment of RC with phosphoprotein phosphatase results in an increased level of apparent autophosphorylation (measured as ^{32}P incorporated). This effect varies with the length of pretreatment. Pretreatment with 1 μM cyclic AMP and the phosphatase greatly enhanced incorporation which may have resulted either from dissociation of phospho-RC or exposure of the seryl phosphate to the phosphatase. Rosen and Erlichman [2], however, found that cyclic AMP also slightly enhanced the rate and degree of autophosphorylation. From both lines of evidence it appears that our enzyme preparations were largely the phospho-enzyme. This may have resulted from an as yet unexplained early loss of phosphoprotein phosphatase activity in our preparation.

Discussion

Autophosphorylation of cyclic AMP-dependent protein kinase has been observed in several tissues [1—4], and it may represent a general property of the cyclic nucleotide-dependent protein kinases. The results of the present study fully accord with the view that this autophosphorylation process occurs via an intramolecular process and the evidence stands against processes involving tetramer-tetramer interactions or even catalytic subunit-tetramer interactions. This implies that the site of the catalytic subunit is proximal to the R-C interface. We propose that the action of the regulatory subunit, in decreasing the activity of the catalytic subunit when bound to it, is similar to the action of trypsin inhibitor; dephospho-R binds in a very affine enzyme-substrate-like complex spanning the protein site. Phospho-R forms an affine enzyme-product complex only in the absence of cAMP.

Witt and Roskoski [11] have used ethoxyformic anhydride inactivation of the brain enzyme in the absence and presence of cyclic AMP as a probe for the topography of the R-C contacts. These workers find that inactivation occurs only in the presence of cyclic AMP for the holoenzyme. $Mg^{2+} \cdot$ ATP protects against inactivation of the free catalytic subunit or of the holoenzyme in the presence of cyclic AMP. Free catalytic subunit can be protected from inactivation by the addition of regulatory subunit. These findings are interpreted in terms of the regulatory subunit acting to shield the active site of the catalytic subunit. It is clear that our findings accord with this proposal.

Rosen and Erlichman [2], in their study on the autophosphorylation of the bovine heart enzyme, found that cyclic AMP stimulated the process only by about two-fold. This small degree of stimulation can be explained in terms of the proposed model by having relatively rapid dissociation of phospho-R subunit only in the presence of cyclic AMP. When these investigators examined the action of protein kinase on heat denatured protein kinase they found that cyclic AMP effected the same degree of stimulation as in the standard assay. This suggests that the slight stimulation of autophosphorylation observed is not due to the release of free catalytic subunits by cyclic AMP and that the molecularity of the autophosphorylation differs from that of the usual protein

kinase reaction in which a bimolecular complexing reaction must precede phosphoryl transfer.

In this study, no evidence has been observed for significant C-C interactions in the tetramer. Further, the kinetics of autophosphorylation are adequately described by models which do not assume such interactions. It is known [10,12] that dissociation of the tetramer by cyclic AMP gives rise to free two equivalents of the catalytic monomer and one equivalent of regulatory dimer, suggesting that C-C interactions in the tetramer may be weak and that the R-R interaction is strong. The proposed model, with the binding sites for the catalytic subunits at opposing ends of the regulatory dimer, is compatible with a weak C-C interaction and a strong R-R one. Support for this idea is found in the reported axial ratios of 12 for both holoenzyme and regulatory dimer and of 3 for the catalytic monomer [13].

Of interest is the variation in the extent of phosphorylation of different preparations of this enzyme. Erlichman et al. [1] report isolating enzyme which is largely dephospho- (and contains a phosphoprotein phosphatase activity). Rosen and Erlichman [1] present data (Fig. 1 of their report) which suggest the kinase exhibits a considerable degree of phosphorylation. Ramseyer et al. [14], using the procedure of Rubin et al. [5] on the porcine muscle system, isolated enzyme which is dissociated on a cyclic AMP affinity column, suggesting, if phosphorylation is prerequisite to cyclic AMP dissociation, that their material is appreciably phosphorylated. Maeno et al. [4], working with the brain enzyme, report autophosphorylation to the extent of 63% and 46% of the regulatory subunits in two sets of experiments. Due to the extreme rapidity of the autophosphorylation reaction [2] under normal assay conditions, variability in the degree of phosphorylation among different preparations of protein kinase may not significantly affect the kinetics of the phosphotransferase reaction.

Lastly, the bovine heart enzyme appears to have a more stable tetrameric structure than the brain enzyme which shows appreciable dissociation upon dilution [10]. The significance of this difference is not clear and requires further investigation.

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