Multiple Activities on Phosphorylase Kinase. 2. Different Specificities toward the Protein Substrates Phosphorylase *b*, Troponin, and Phosphorylase Kinase†

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ABSTRACT: Phosphorylase kinase exhibits three kinds of enzymatic activities. A partial activity, A_0, catalyzes the phosphorylation of phosphorylase *b*, troponin I, and phosphorylase kinase itself (autophosphorylation); A_1 can utilize only phosphorylase *b* and phosphorylase kinase as the substrate, whereas A_2 can utilize only phosphorylase *b* and troponin T. Stimulation of A_1 by Ca^{2+} coincides with an increase in the number of sites that can undergo self-phosphorylation ranging from ca. 35 to ca. 70 mol of phosphate incorporated/1.28 × 10^6 g of proteins. Inhibition of A_0 and A_1 by millimolar Ca^{2+} is accompanied by a decrease in substrate availability during self-phosphorylation. NH_4Cl (150 mM) strongly inhibits the availability of troponin as a substrate. In the course of self-phosphorylation, the activities A_0 and A_1 are both stimulated moderately by an increase in pH; however, only A_1 shows some inhibition by 150 mM NH_4Cl. Millimolar Ca^{2+} inhibits A_1 and A_2 as measured by self-phosphorylation or troponin phosphorylation, as observed with the phosphorylation of phosphorylase *b* [Kilimann, M. W., & Heilmeyer, L. M. G., Jr. (1982) *Biochemistry* (preceding paper in this issue)]. The rate of self-phosphorylation varies as a function of substrate concentration (K_m = 68 nM at 10 mM Mg^{2+} and 184 μM Ca^{2+}, pH 9.0). The data indicate that both Ca^{2+} activation and inhibition seem to be mediated by phosphorylase kinase itself rather than by the substrates.

In addition to utilizing phosphorylase *b* as a substrate, phosphorylase kinase catalyzes its own phosphorylation, modifying its α and β subunits self-phosphorylation (Krebs et al., 1959, 1964). Furthermore, troponin T (the tropomyosin binding subunit of troponin), troponin I (the inhibitory component) [Walsh et al., 1971; cf. Sperling et al. (1979)], and the N-terminal region of glycogen synthase (Roach et al., 1978; De Paoli-Roach et al., 1979; Embi et al., 1979) can serve as the substrate. In the preceding paper, three partial activities of phosphorylase kinase, designated as A_0, A_1, and A_2, were characterized by their response to Ca^{2+}, Mg^{2+}, NH_4Cl, and pH; their behavior upon phosphorylation or partial proteolysis of the enzyme was also described.

The question was raised whether these three partial activities could also be differentiated by the use of the alternate protein substrates mentioned above. Could they serve to reveal whether the changes in phosphorylase kinase activity observed as a function of the various effectors could be mediated by phosphorylase kinase or its protein substrates? This publication reports that, indeed, most of the observed effects are mediated by the enzyme itself. In addition, it shows that the specificity toward the various protein substrates can also serve to differentiate the three partial activities A_0, A_1, and A_2.

Experimental Procedures

Phosphorylase kinase activity toward troponin and during self-phosphorylation was measured by combining 50 μL of troponin P^1 TI_2C (10–12 mg/mL in 1.5 mM sodium glycerol 2-phosphate and 0.75 mM dithioerythritol, free of contaminating proteases) as prepared according to Sperling et al. (1979), 50 μL of ATP/Mg^{2+} (containing 17 mM [γ-^{32}P]ATP, ca. 50 Ci/mol, and 41–47 mM $MgCl_2$), 25 μL of medium buffer, and 25 μL of kinase. Each solution was adjusted to the required pH immediately prior to the assay. For self-phosphorylation, troponin-free buffer was used. The medium buffer (1.5 mM sodium glycerol 2-phosphate, 0.75 mM dithioerythritol, and 6 mM EGTA) also served to adjust the NH_4Cl and free Ca^{2+} concentrations. Phosphorylase kinase (1 mg/mL) was dissolved in 1.5 mM sodium glycerol 2-phosphate and 0.75 mM dithioerythritol, pH 6.8. The reagents were mixed within 60 s and the reaction was started by addition of the enzyme. The pH of the medium buffer, the troponin, and the ATP/Mg^{2+} solutions was identical with that of the assay mixture at all Ca^{2+} concentrations. For determination of the initial rate of phosphate incorporation, aliquots were removed at t = 0, 50, 100, and 150 s. Protein-bound phosphate was determined according to Mans & Novelli (1961); uptake was linear during the time course of the reaction. One unit of activity is defined as 1 μmol of phosphate transferred min^{-1} (mg of enzyme)$^{-1}$. Troponin phosphorylation, which is slow, was corrected for phosphate uptake into the enzyme itself. Polyacrylamide gel (5% for phosphorylase kinase; 10% for troponin) electrophoresis in the presence of 1% sodium dodecyl sulfate (Weber & Osborn, 1969) showed the same extent of phosphate incorporation into the α and β subunits whether troponin was present or not, despite the fact that the concentrations of both substrates are in the range of their respective K_m values [see Dickneite et al. (1978) and below]. The gels were sliced in a Gilson gel fractionator, and the distribution of radioactivity was determined by liquid scintillation counting.

In addition to the association constants reported earlier (Kilimann & Heilmeyer, 1982), those for troponin were as follows: two Ca^{2+} bound to the Ca–Mg sites, 8.72; two Mg^{2+} bound to the Ca–Mg sites, 3.60; two Ca^{2+} bound to the Ca-specific sites, 6.69 [all logarithmic values (Potter & Gergely, 1975)].

Troponin was dialyzed for 16 h against 50 volumes of 1.4 mM sodium glycerol 2-phosphate and 0.75 mM dithioerythritol, pH 6.8; solutions of 10 mg/mL contained 0.5–0.8 mM Ca^{2+}. Phosphorylase kinase was filtered over Sephadex G-50 superfine in the same buffer: solutions of 1 mg/mL contained ca. 10 μM Ca^{2+}. For troponin phosphorylation and

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Table I: Ca^{2+} Dependence of Autophosphorylation[a]

pH	ionic strength (mM)	initial velocity V_0 (mU/mg)	initial velocity V_1 (mU/mg)	initial velocity $K'_{m,1}$ (μM)	initial velocity $K'_{i,1}$ (mM)	saturating phosphate incorpn (50 min) incorpn$_0$ (mol/mol)	incorpn$_1$ (mol/mol)	$K'_{m,1}$ (μM)	$K'_{i,1}$ (mM)
6.8	50	–	–	–	–	6.9	15.4	0.86	6.1
	200	0.21	1.43	0.81	5.9	–	–	–	–
9.0	50	1.41	5.7	1.56	4.2	26.5	36.3	0.45	5.9
	200	1.21	3.4	1.90	2.2	16.1	33.9	1.25	4.2

[a] Measurements at pH 6.8 were carried out at 25 °C; pH 9.0 measurements were at 30 °C. The indices in the parameter denominations denounce whether they belong to the activities A_0 or A_1. The Ca^{2+} dependence of the saturating phosphate incorporation was treated in calculation like the initial velocity; here, the values designated as K'_m and K'_i are the Ca^{2+} concentrations of half-maximal increase and decrease of the degree of phosphorylation.

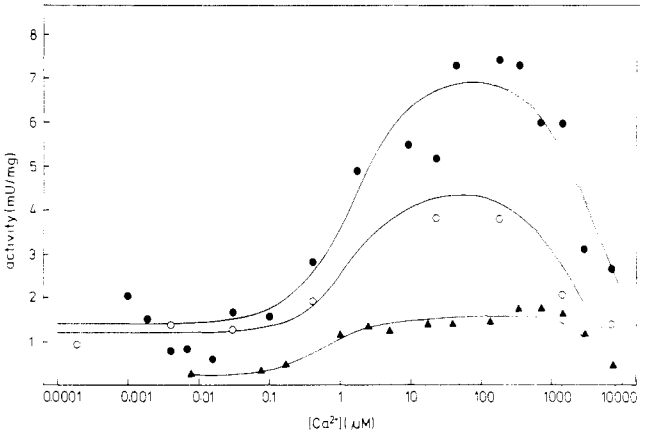

FIGURE 1: Ca^{2+} and NH_4Cl dependence of the initial velocity of autophosphorylation. Phosphorylation was carried out at (▲) pH 6.8, 7.4 mM Mg^{2+}, 150 mM NH_4Cl, 0.45 mg/mL phosphorylase kinase, and 26 °C, (○; ●) pH 9.0, 30 °C, and 0.20 mg/mL phosphorylase kinase in the presence (○) or absence (●) of 150 mM NH_4Cl; the free Mg^{2+} concentration was between 6.8 mM at the lowest and 8.6 mM at the highest Ca^{2+} concentration, due to competition between Mg^{2+} and Ca^{2+} for chelation by ATP and EGTA. Curves are computer fitted.

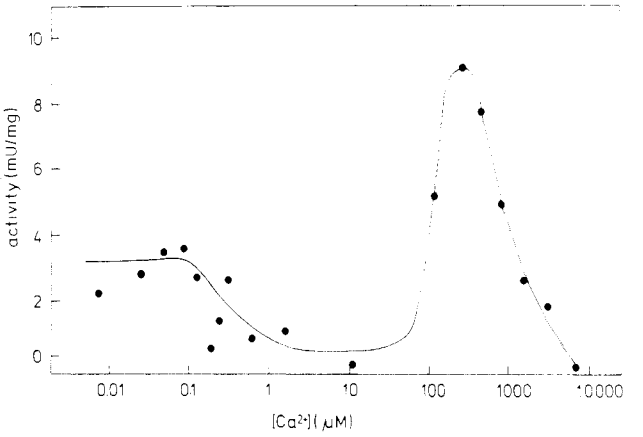

FIGURE 2: Ca^{2+} dependence of the initial velocity of troponin phosphorylation at pH 9.0. Measurements were carried out at 30 °C in the absence of NH_4Cl. 0.20 mg/mL phosphorylase kinase and 4.0 mg/mL troponin were contained in the assay; the free Mg^{2+} concentration was between 7.0 mM at the lowest and 8.4 mM at the highest Ca^{2+} concentration, due to competition between Ca^{2+} and Mg^{2+} for chelation by ATP and EGTA.

self-phosphorylation measurements, both proteins were transferred to the assay buffer since the solution in which the enzyme is stored is highly inhibitory. [γ-^{32}P]ATP was prepared according to Glynn & Chappell (1964). All other materials and methods were described in Kilimann & Heilmeyer (1982).

Results

The initial velocity of phosphorylase kinase self-phosphorylation at pH 6.8 is both stimulated and inhibited by Ca^{2+} in the micromolar and millimolar range, respectively (Figure 1). The apparent K'_m and K'_i values are similar to those found for the activity A_1 when phosphorylase *b* is used as the substrate (Table I). Below approximately 10 nM Ca^{2+}, one observes another partial activity, presumably A_0. Over the whole Ca^{2+} concentration range, A_2 cannot be detected even at pH 9.0. Table I shows that addition of 150 mM NH_4Cl decreases the V_{max} of A_1 while A_0 is not significantly affected.

At pH 9.0 the kinase activity toward troponin exhibits a finite level between ca. 7 and 100 nM Ca^{2+} (Figure 2) which may be attributed once more to A_0. Above 0.1 μM Ca^{2+} the activity decreases significantly before leveling off from 0.6 to 10 μM Ca^{2+}. In this Ca^{2+} concentration range the activity A_1 is expressed with phosphorylase *b* as the substrate (K'_m = 1 μM Ca^{2+}; see preceding paper); with troponin around 1 μM Ca^{2+} an inhibition is even found. At Ca^{2+} concentrations above 10 μM, the activity increases ca. 20-fold and then goes through a sharp maximum around 200 μM Ca^{2+} that is characteristic for A_2. Addition of 150 mM NH_4Cl leads to a 20–50% decrease in activity over the whole Ca^{2+} concentration range (not shown).

The time courses of troponin phosphorylation and self-phosphorylation were determined at various calcium concentrations, namely, (a) at ca. 5 nM Ca^{2+} where only A_0 is expressed, (b) at ca. 1 μM Ca^{2+}, i.e., where an inflection point is observed for increased self-phosphorylation or decreased troponin phosphorylation, and (c) at ca. 150 μM Ca^{2+} where A_1 and A_2 display maximum activity (Figure 3). In all instances, the autocatalytic phosphate incorporation is terminated after ca. 60 min. The degree of phosphorylase kinase phosphorylation (moles per mole) does not increase with increasing enzyme concentration (not shown). However, at 3.9 nM Ca^{2+}, only half the amount of phosphate is incorporated by A_0 as opposed to the two other Ca^{2+} concentrations at which A_1 is expressed. Only the α and β subunits are phosphorylated in accordance with earlier observations (Hayakawa et al., 1973; Hörl et al., 1975; Wang et al., 1976). The relative distribution of phosphate at saturation between these subunits is not significantly influenced by Ca^{2+} (not illustrated).

Phosphate incorporation into troponin is completed after 60–90 min at the lowest and highest Ca^{2+} concentrations of 5 nM and 150 μM, respectively. However, the amount incorporated falls by ca. 2.8-fold with decreasing free Ca^{2+} concentration; this decrease coincides with a shift from troponin T to troponin I as the best phosphate acceptor (Table II). At the intermediate Ca^{2+} concentration of 1 μM, phosphorylation still proceeds after 120 min and, presumably, will reach the same level as observed with 150 μM Ca^{2+} (see Figure 3).

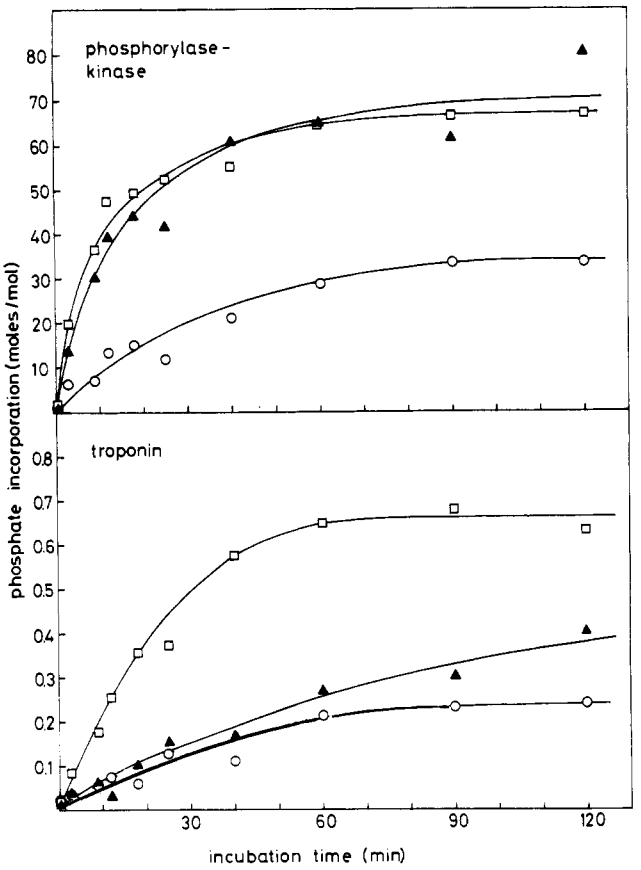

FIGURE 3: Time course of autophosphorylation and troponin phosphorylation at pH 9.0 in dependence of Ca^{2+} concentration. Measurements were carried out at 30 °C. 0.155 mg/mL phosphorylase kinase and, if applicable, 4.0 mg/mL troponin were contained in the assays, but no NH_4Cl was contained. The Ca^{2+} and Mg^{2+} concentrations were as follows: (○) phosphorylase kinase, 3.9 nM Ca^{2+} and 7.0 mM Mg^{2+}; troponin, 7.3 nM Ca^{2+} and 7.0 mM Mg^{2+}; (▲) phosphorylase kinase, 1.7 μM Ca^{2+} and 7.7 mM Mg^{2+}; troponin, 0.61μM Ca^{2+} and 7.7 mM Mg^{2+}; (□) phosphorylase kinase, 184 μM Ca^{2+} and 7.8 mM Mg^{2+}; troponin, 124 μM Ca^{2+} and 7.7 mM Mg^{2+}. Phosphate incorporation is given in moles per mole of holoprotein (1.28 × 10^6 g of phosphorylase kinase; 9 × 10^4 g of troponin).

Table II: Ca^{2+} Dependence of Phosphate Incorporation into the Troponin Subunits[a]

time (min)	Ca^{2+} (μM)	phosphate incorpn (mol/90 000 g of troponin) Tn-I	Tn-T	total
6	0.0073	0.032	0.013	0.045
	0.61	0.018	0.032	0.049
	124	0.017	0.121	0.138
60	0.0073	0.145	0.066	0.211
	0.61	0.071	0.183	0.254
	124	0.056	0.591	0.647

[a] Experimental details are given in the legend to Figure 3. No phosphate incorporation into the Tn-C subunit was observed.

The total amount of phosphate incorporated during self-phosphorylation is low below 0.1 μM Ca^{2+}. It more than doubles at or above 10 μM Ca^{2+} and decreases again above 500 μM Ca^{2+} (Figure 4). Table I shows that the Ca^{2+} concentrations at which the half-maximal effects are observed correspond to the K'_m(Ca) and K'_i(Ca) values for A_1. NH_4Cl (150 mM) reduces the phosphate incorporation to a minor degree in the whole Ca^{2+} concentration range (Table I).

The degree of troponin phosphorylation was investigated at Ca^{2+} concentrations between 0.1 and 400 μM. At pH 6.8, phosphate uptake varies between 0.07 and 0.13 mol/mol with a shallow minimum observed around 0.3 μM Ca^{2+} (not illustrated). At pH 9.0 a constant amount of approximately 0.13 mol/mol is incorporated between 7 and 100 nM Ca^{2+} (Figure 5). Above this concentration, phosphorylation increases ca. 8-fold, yielding a wide peak with maximum around 120 μM Ca^{2+}. Addition of 150 mM NH_4Cl leads to a strong reduction in phosphate incorporation (Figure 5).

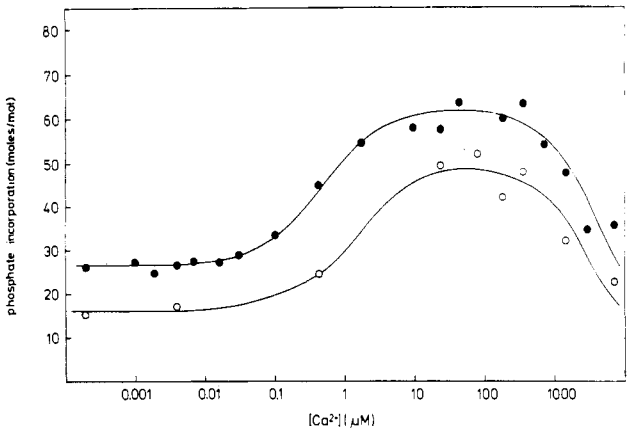

FIGURE 4: Ca^{2+} dependence of saturating autocatalytic phosphate incorporation at pH 9.0. Phosphorylation was carried out at conditions analogous to those in Figure 1 [experiments (●) and (○)]; given values are means of two samples taken after 45 and 60 min. Curves are computer fitted.

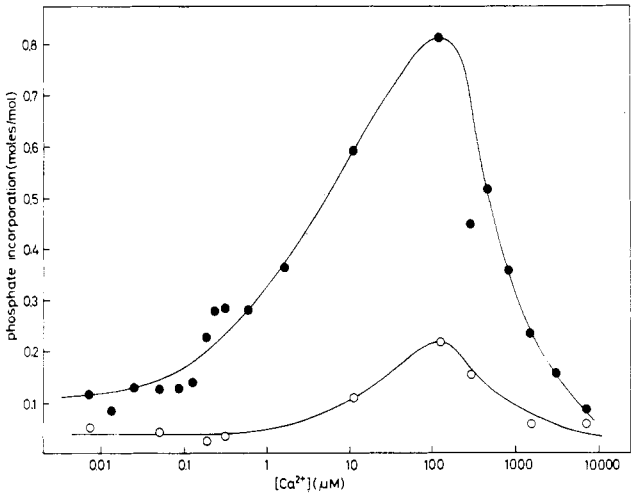

FIGURE 5: Ca^{2+} dependence of phosphate incorporation into troponin after 48 min at pH 9.0. (○) 150 mM NH_4Cl; (●) without NH_4Cl. Other assay conditions are described in the legend to Figure 2.

Self-phosphorylation which can be catalyzed only by A_0 and A_1 in the 100–300 μM Ca^{2+} range increases moderately from pH 6.0 to pH 9.0 and is slightly inhibited by salts (Figure 6). These two effects are observed as well when phosphorylase *b* is used as the substrate. Troponin phosphorylation is markedly stimulated by an increase in pH at the same free Ca^{2+} concentrations and inhibited by 150 mM NH_4Cl more sharply than self-phosphorylation is. The pH effect can be explained by the induction of A_2, whereas the strong salt inhibition becomes evident mainly in the degree of phosphorylation after 50 min, which probably can be attributed to a reduction of troponin availability as the substrate.

Self-phosphorylation of phosphorylase kinase was assayed as a function of enzyme concentration (from 0.03 to 0.15 μM; enzyme of M_r 1.28 × 10^6) in 7.8 mM Mg^{2+}, 184 μM Ca^{2+}, and pH 9.0. The initial velocity increases rapidly with kinase concentration. For elimination of effects of dilution, the activities obtained were multiplied by their dilution factors. The resulting values still decrease hyperbolically with kinase concentration, reflecting a dependence of activity not only on the

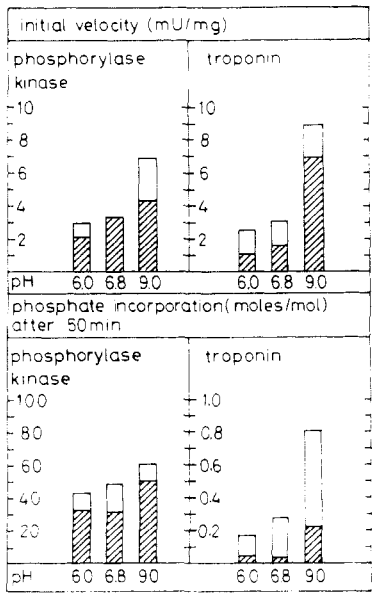

FIGURE 6: Dependence of autocatalytic and troponin phosphorylation at 200 μM Ca^{2+} from pH and salt concentration. Measurements were carried out at 30 °C. 0.20 mg/mL phosphorylase kinase and, if applicable, 4.0 mg/mL troponin were contained in the assays. The given values are means of two or three determinations at Ca^{2+} concentrations between 100 and 300 μM Ca^{2+}. The Mg^{2+} concentrations were 8.2 mM at pH 6.0, 7.9 mM at pH 6.8, and 7.8 mM at pH 9.0. The total height of a bar represents the activity or the degree of phosphorylation, respectively, in the absence and its hatched part that in the presence of 150 mM NH_4Cl.

amount of enzyme present as catalyst but also on the amount of enzyme present as substrate. For the latter effect, an apparent K_m value of 60 ± 16 nM was calculated.

Discussion

When a protein kinase acts on a protein substrate, it is sometimes difficult to decide whether the observed effects are due to modifications of the catalyst or the substrate. In this paper, we try to distinguish between these alternatives. The matter is complicated by the fact that both enzyme and the substrate (troponin) can be phosphorylated and bind Ca^{2+}.

The A_0 activity of phosphorylase kinase phosphorylates all three protein substrates tested, namely, phosphorylase *b*, troponin I [see also Sperling et al. (1979)], and the enzyme itself (autocatalysis). By contrast, activity A_1 acts only on phosphorylase *b* and phosphorylase kinase and A_2 only on phosphorylase *b* and troponin T. During self-phosphorylation, the increase and decrease of the total amount of phosphate incorporated parallel the extent of activation and inhibition of A_1 by Ca^{2+}, respectively. A_1 also decreases upon addition of NH_4Cl or by lowering the pH (Table I). Two hypotheses can account for these observations, namely (a) that different numbers of sites susceptible to being phosphorylated are exposed under these different conditions or (b) that the basal activity A_0, expressed independently of Ca^{2+} concentration, exhibits a more restricted substrate specificity in that it can phosphorylate only a few of the serine residues normally hit by A_0 and A_1. In any event, the sites susceptible to self-phosphorylation can be subdivided into two classes: one which is phosphorylated below 0.1 μM Ca^{2+} by A_0 and the other above 1 μM Ca^{2+} by A_1. This latter group can include the former, though not necessarily. The relative initial rate of self-phosphorylation varies appreciably (3–5-fold) as a function of Ca^{2+}, NH_4Cl, and pH. This variation cannot be accounted for on the basis of nonavailability of sites since the total amount of phosphate incorporated varies by only 2-fold under these conditions. Furthermore, the substrate concentration always remains well above K_m (see below). These three effectors thus appear to affect the properties of the enzyme in its role as a catalyst rather than as a substrate.

The I subunit of the troponin complex is the component predominantly phosphorylated at low Ca^{2+} concentration. Increase of Ca^{2+} leads to a reduction of troponin I and an increase in troponin T phosphorylation [see Table II and Sperling et al. (1979)] which could result from a change in conformation and subsequent alteration in subunit interaction within the troponin complex [for a discussion, see Sperling et al. (1979)].

Below 0.1 μM Ca^{2+}, only activity A_0 is expressed. Because of the decreased availability of troponin I as the substrate at $[Ca^{2+}] > 0.1$ μM, its initial rate of phosphorylation is reduced (Figure 2). By contrast, availability of troponin T as the substrate increases (Table II), but since it is phosphorylated at a lower initial velocity, saturation was not reached within the time course of the reaction (48 min), yielding the asymmetric peak seen in Figure 5. No stimulation of A_1 between 0.1 and 10 μM Ca^{2+} can be observed. In this range of concentration where activity A_0 vanishes because of decreased substrate availability, the presence of traces of A_1 activity cannot be completely excluded; however, if some is expressed, its contribution to the total activity would be minimal. Above 50 μM Ca^{2+}, A_2 is expressed as illustrated in Figure 2 from its characteristic dependence on Ca^{2+} and pH (compare with preceding paper). Only the phosphorylation of troponin T is stimulated following the appearance of A_2 while the residual phosphorylation of troponin I remains unaffected (Table II).

Inhibition of A_0 and A_1 by millimolar Ca^{2+} is observed whether phosphorylase *b* or the kinase itself is used as the substrate (Figure 1). The same applies to activity A_2 with phosphorylase *b* and troponin T (Figure 2). This effect, therefore, cannot possibly be attributed to Ca^{2+} binding to any of the protein substrates (see also preceding paper) and, therefore, must affect the enzyme. A similar conclusion can be drawn from the inhibition of A_1 by NH_4Cl, which is observed when phosphorylase *b* is present as the substrate as well as during self-phosphorylation, whereas A_0 is not affected in either case.

At a concentration of kinase above 1 μM, the rate of self-phosphorylation is independent of substrate concentration, which might suggest an intramolecular reaction (G. Dickneite and L. M. G. Heilmeyer, Jr., unpublished experiments). Below 1 μM self-phosphorylation shows a hyperbolic substrate dependence. This could result either from dissociation and concomitant inactivation of the enzyme or from self-phosphorylation occurring intermolecularly under these conditions. Such an intermolecular reaction could well be stimulated by other proteins such as phosphorylase *b*, peptide substrates, and nonsubstrate proteins as described by Carlson & Graves (1976). It is certainly remarkable that the apparent K_m for self-phosphorylation is almost 3 orders of magnitude lower than the K_m values for both phosphorylase *b* and troponin (Krebs et al., 1964; Dickneite et al., 1978).

The observations that A_0, A_1, and A_2 can be controlled independently by ionic effectors as well as by covalent modifications (see preceding paper) and, especially, that these activities display different substrate specificities could be readily explained if one assumed the existence of different catalytic centers on the enzyme. The decrease in K_m toward phosphorylase *b* with increasing pH (Krebs et al., 1964) or after phosphorylation by cAMP-dependent protein kinase (DeLange et al., 1968) could then be understood if the catalytic center expressing A_2 had a higher affinity for this substrate

than that expressed by A_0 or A_1. By analogy, the decrease in K_m for phosphorylase *b* when Ca^{2+} is lowered from 10^{-6} to 10^{-8} M (Heilmeyer et al., 1970) could be explained on the basis of different substrate affinities for the group responsible for the A_0 and A_1 activity.

The suggestion that phosphorylase kinase might possess more than one catalytic center was first advanced in a study of the inhibition of phosphorylase *b* and troponin phosphorylation by immunoglobulins directed against phosphorylase kinase (Dickneite et al., 1978). Similar conclusions were drawn from an affinity labeling study of the enzyme with reactive ATP analogues. Covalent modifications occurred stoichiometrically at two sites on the β subunit and at one site on the γ subunit (Gulyaeva et al., 1978). Recently, inactivation of phosphorylase kinase with the 2′,3′-dialdehyde derivative of ATP (oATP) also tended to indicate the presence of more than one catalytic site on the enzyme: one preferentially involved in the phosphorylation of glycogen synthase and phosphorylase *b* and the other troponin I and troponin T (King & Carlson, 1981 a,b). In fact, the existence of several catalytic centers located on different subunits may resolve the uncertainty regarding the identity of the catalytic subunit in phosphorylase kinase, in which the β (Fischer et al., 1978) as well as the γ subunits (Skuster et al., 1980) have been implicated. However, a third though less likely possibility, namely, that the enzyme contains only one catalytic center whose activity increases step by step in going from A_0 to A_1 to A_2 with increasing Ca^{2+} concentration, cannot be ruled out.

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