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Interaction of Phosphorylase Kinase with the 2′,3′-Dialdehyde Derivative of Adenosine Triphosphate. 2. Differential Inactivation Measured with Various Protein Substrates†

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ABSTRACT: The 2′,3′-dialdehyde derivative of ATP was used as an affinity label to inactivate phosphorylase kinase in either the presence or absence of Ca^{2+} and Mg^{2+} ions. Following inactivation, the residual activity of phosphorylase kinase toward various protein substrates was measured and compared with that retained for conversion of phosphorylase *b*. Three different classes of substrates were distinguished by this analysis. For the first class (glycogen synthase), inactivation proceeded at the same rate as that measured with phosphorylase conversion, regardless of whether the inactivation was carried out in the presence or absence of the metal ions. For the second class of substrates (troponin I and troponin T), inactivation of the kinase in either the presence or absence of the metals was much more rapid with phosphorylase as substrate. Phosphorylation of the third class of substrates (phosphorylase kinase itself and a synthetic tetradecapeptide) was inactivated in parallel with phosphorylase *b* when modification was performed in the absence of metals; however, inclusion of Ca^{2+} and Mg^{2+} ions in the inactivation mixture caused activity toward phosphorylase *b* to be lost more rapidly than that toward the alternative substrates. Our results are consistent with a model in which glycogen synthase and phosphorylase *b* are preferentially phosphorylated at one type of catalytic site in phosphorylase kinase and troponin I and troponin T at another.

Phosphorylase *b* kinase (ATP:phosphorylase-*b* phosphotransferase, EC 2.7.1.38) of rabbit skeletal muscle is a hexadecamer composed of four different types of subunits, $\alpha_4\beta_4\gamma_4\delta_4$ (Cohen et al., 1978). The enzyme catalyzes the phosphorylation of phosphorylase *b* as well as the phosphorylation of itself (DeLange et al., 1968), troponin I (Stull et al., 1972), troponin T (Perry & Cole, 1974), and glycogen synthase (Roach et al., 1978).

There is not general agreement, however, as to which subunit(s) catalyzes (catalyze) the above reactions, or indeed, if phosphorylation of all the protein substrates is even carried out by a single type of catalytic site. Both the β and γ subunits have been reported to have catalytic capacity. Skuster et al. (1980) reported the isolation of catalytically active γ subunit after dissociation of the enzyme with LiBr. Gulyaeva et al.

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(1977), using a series of alkylating ATP affinity labels, found that loss of catalytic activity parallelled modification of the β subunit. Further evidence suggesting that the β subunit has catalytic capability was provided by Fischer et al. (1978), who found a catalytically active, radioactive peptide fragment after chymotryptic digestion of phosphorylase kinase that had been labeled with ^{32}P predominantly in the β subunit.

The possibility that both subunits may be active in the holoenzyme is supported by literature data that is consistent with more than one type of catalytic site in the holoenzyme. Relying upon inhibition studies, Dickneite et al. (1978) proposed that there are two separate catalytic sites for the phosphorylation of phosphorylase *b* and troponin T. Two types of sites are also consistent with the paradox reported by Carlson & Graves (1976), who found that autophosphorylation of phosphorylase kinase was not inhibited by apparently saturating concentrations of an octapeptide substrate or a hexapeptide inhibitor of phosphorylase conversion. For a more complete review of this general question, see Carlson et al. (1979).

In this report we examine the question of whether there is more than one type of catalytic site in phosphorylase kinase by comparing the loss of enzymatic activity toward various protein substrates following modification of the kinase with an ATP affinity label. The substrates compared after modification of the enzyme with the 2′,3′-dialdehyde of ATP (oATP)[1] were phosphorylase kinase itself, glycogen synthase, troponin I, troponin T, phosphorylase *b*, and a peptide corresponding to its phosphorylation site. Our results are consistent with the presence of more than one type of catalytic site in phosphorylase kinase.

Materials and Methods

Purification, characterization, and assays of nonactivated phosphorylase kinase and phosphorylase *b* were performed as described in the preceding paper in this issue (King & Carlson, 1981), as was the preparation of phosphorylated phosphorylase kinase. The following individuals generously supplied these proteins purified from rabbit skeletal muscle: glycogen synthase, Thomas Soderling and Balwant Khatra, Vanderbilt University; troponins I and T, Marion Greaser, University of Wisconsin. Synthetic tetradecapeptide was a gift from Donald Graves, Iowa State University. In the absence of phosphorylase kinase, no ^{32}P was incorporated from [γ-^{32}P]ATP into any of these substrates, indicating that all these proteins were free of interfering kinases. In addition, assays measuring phosphorylation by phosphorylase kinase of itself, phosphorylase *b*, glycogen synthase, and troponin I were nearly totally inhibited by addition of EGTA to the assay mixtures, thus indicating the absence of interfering kinases in the phosphorylase kinase preparation. Phosphorylation of troponin T at pH 8.2 was only inhibited 75% by addition of EGTA to the assays, although when β-glycerophosphate, a known effector of phosphorylase kinase (Wang et al., 1976; Carlson & Graves, 1976), was included with the EGTA, the inhibition was virtually total.

^{32}P incorporation into the tetradecapeptide utilized a phosphocellulose paper assay (Tessmer et al., 1977). Throughout this report we made certain that residual activity was being determined within the initial linear portion of the time course of phosphorylation of the various substrates. All other reagents and procedures were as described in the preceding paper (King & Carlson, 1981).

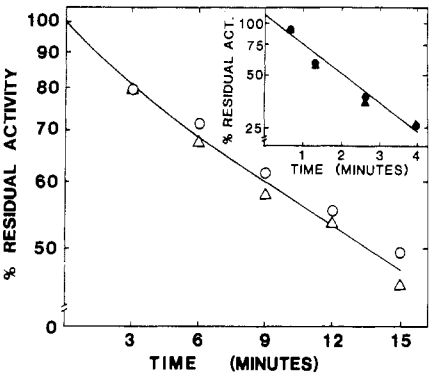

FIGURE 1: Comparison of the effect of oATP on the phosphorylation of phosphorylase *b* and glycogen synthase. Inactivation in the absence of Ca^{2+} and Mg^{2+}. Nonactivated phosphorylase kinase (0.25 mg/mL) was inactivated at 30 °C by oATP (0.1 mM) in Hepes buffer (35 mM, pH 6.8) containing EDTA (0.02 mM). At the indicated times, aliquots were diluted 50-fold with cold buffer (40 mM β-glycerophosphate and 30 mM mercaptoethanol, pH 6.8) and then diluted 6-fold into an assay mixture containing, in final concentrations, kinase (1 μg/mL), phosphorylase *b* (2 mg/mL) or glycogen synthase (0.25 mg/mL), MgATP [10 mM $Mg(CH_3CO_2)_2$ and 0.8 mM [^{32}P]ATP at 31 Ci/mol], $CaCl_2$(0.24 mM), EDTA (6 μM), KP_i (1.6 mM), sucrose (3.8%), mercaptoethanol (12 mM), and buffer (50 mM Tris and 63 mM β-glycerophosphate, pH 8.2). After 4 min at 30 °C, aliquots were removed for determination of ^{32}P incorporation into protein. (○) Phosphorylase *b*; (△) glycogen synthase. (Inset) Inactivation in the presence of Ca^{2+} and Mg^{2+}. Nonactivated phosphorylase kinase (0.25 mg/mL) was inactivated at 30 °C by oATP (10 μM) in Hepes buffer (27 mM, pH 6.8) containing $CaCl_2$ (0.4 mM), $Mg(CH_3CO_2)_2$ (10 mM), EGTA (0.15 mM), EDTA (0.02 mM), and sucrose (1%). At the indicated times, aliquots were diluted 41-fold with cold buffer (40 mM β-glycerophosphate and 30 mM mercaptoethanol, pH 6.8) and diluted 6-fold into an assay mixture containing, in final concentrations, kinase (1 μg/mL), phosphorylase *b* (2 mg/mL) or glycogen synthase (0.28 mg/mL), MgATP [10 mM $Mg(CH_3CO_2)_2$ and 0.8 mM [^{32}P]ATP at 24.4 Ci/mol], $CaCl_2$ (0.25 mM), EDTA (0.17 mM), KP_i (4.2 mM), sucrose (10%), mercaptoethanol (11.8 mM), and buffer (52 mM Tris and 61 mM β-glycerophosphate, pH 8.2). After 4 min at 30 °C, aliquots were removed for determination of ^{32}P incorporation into protein. (●) Phosphorylase *b*; (▲) glycogen synthase.

Results

Comparison of the Effect of oATP on the Phosphorylation of Glycogen Synthase and Phosphorylase b. The characterization of the interaction of the enzyme with oATP provided evidence that oATP is a valid ATP affinity label apparently directed toward the active site of phosphorylase kinase (King & Carlson, 1981). We therefore chose to utilize oATP as a tool to investigate whether there might be more than one type of catalytic site in phosphorylase kinase. If two protein substrates are phosphorylated at the same catalytic site, then only one rate of inactivation by oATP should be observed. If, however, there are two different types of catalytic sites with different preferences for various protein substrates, then the inactivation curves with these various proteins as substrates should not be superimposable, provided that the two sites either have different binding affinities for the oATP or react with it at different rates.

Because phosphorylase *b* is the most readily available protein substrate of phosphorylase kinase, we chose to compare the effect of oATP on phosphorylase conversion with the effects of oATP on the phosphorylation of other substrates. Glycogen synthase is the first substrate for which such a comparison is

[1] Abbreviations used: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; AMPPNP, adenylyl β,γ-imidodiphosphate; oATP, 2′,3′-dialdehyde of ATP; oAMPPNP, 2′,3′-dialdehyde of adenylyl β,γ-imidodiphosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

shown (Figure 1). Inactivation in Figure 1 was carried out with 100 μM oATP in the absence of Ca^{2+} and Mg^{2+}. The residual activity was then determined with phosphorylase *b* and glycogen synthase as the substrates. The inset shows an experiment in which inactivation was carried out with 10 μM oATP in the presence of Ca^{2+} and Mg^{2+}. Under both conditions, the rate of inactivation was the same for these two substrates, suggesting that phosphorylase *b* and glycogen synthase are phosphorylated at the same site on phosphorylase kinase.

Because phosphorylase *b* is such a good substrate, it was necessary to use higher assay concentrations of it than those used for the other protein substrates in this report in order to ensure that data were being collected in linear regions of the phosphorylation time course. Routine use of lower concentrations of phosphorylase *b* would have been technically impractical. However, we did carry out control experiments in which the concentration of phosphorylase *b* in the assay was either 0.4 or 4.0 mg/mL in order to be certain that the concentration of protein substrate in the assay had no effect on the observed rate of inactivation by oATP. The higher concentration of substrate approximated previously reported values for the K_m of phosphorylase *b* (Krebs et al., 1964; Stull et al., 1972) whereas the lower concentration was well below the K_m value. Under otherwise identical conditions, the two concentrations of phosphorylase *b* showed the same rate of inactivation by oATP (data not shown), which justified the use of the higher concentration of phosphorylase in this work.

Comparison of the Effect of oATP on the Phosphorylation of Phosphorylase b and a Tetradecapeptide. The tetradecapeptide Ser-Asp-Gln-Glu-Lys-Arg-Lys-Gln-Ile-Ser-Val-Arg-Gly-Leu corresponds to residues 5–18 of phosphorylase *b*. The peptide and phosphorylase *b* are phosphorylated by phosphorylase kinase exclusively at the seryl residue between Ile and Val (Tessmer & Graves, 1973). Furthermore, it has been shown that the peptide behaves like phosphorylase *b* during various phosphorylation assays with phosphorylase kinase (Carlson et al., 1975). This suggested that the peptide substrate was a valid low-molecular-weight model of phosphorylase *b*.

It seemed reasonable to assume that phosphorylase *b* and the tetradecapeptide would be phosphorylated at the same site on phosphorylase kinase and that inactivation by oATP would show one rate of inactivation with these two substrates. This was found to be the case when inactivation of phosphorylase kinase was carried out with 50 μM oATP in the absence of Ca^{2+} and Mg^{2+} (Figure 2A). Phosphorylase *b* and the tetradecapeptide showed only one rate of inactivation by oATP. However, when inactivation by oATP (10 μM) was carried out in the presence of the two metal ions (Figure 2B), two rates of inactivation were observed with the two substates. Inactivation measured with phosphorylase *b* was more than 2-fold faster than that measured with the tetradecapeptide. Thus, we have the paradoxical observation of an identical rate of inactivation under one set of conditions and different rates of inactivation under another condition. That this difference in results is not just due to the necessary difference in the concentration of inhibitor, or to the inclusion of Ca^{2+} and Mg^{2+}, will be obvious from the results obtained with troponin I and T.

Comparison of the Effect of Inhibitor on the Conversion of Phosphorylase b and Autophosphorylation. When the rate of inactivation by 100 μM oATP was measured by autophosphorylation and compared to the rate measured by phosphorylase *b* conversion, one rate was observed when the

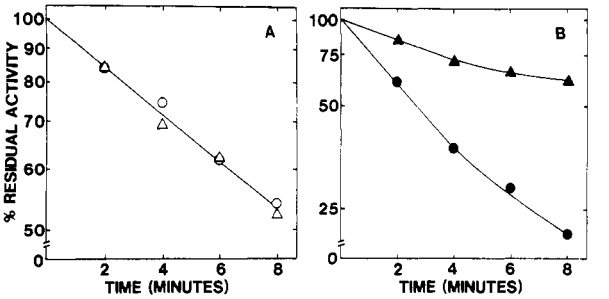

FIGURE 2: Comparison of the effect of oATP on the phosphorylation of phosphorylase *b* and the tetradecapeptide. (A) Inactivation in the absence of Ca^{2+} and Mg^{2+}. Nonactivated phosphorylase kinase (0.18 mg/mL) was inactivated at 30 °C by oATP (50 μM) in Hepes buffer (33 mM, pH 6.8) in the presence of sucrose (0.7%) and EDTA (15 μM). For determination of the residual activity at the indicated times with phosphorylase *b*, aliquots were diluted 100-fold with cold buffer (40 mM Hepes and 20 mM mercaptoethanol, pH 6.8). These samples were then diluted 5-fold into an assay mixture containing, in final concentrations, kinase (0.36 μg/mL), phosphorylase *b* (5 mg/mL), MgATP [10 mM $Mg(CH_3CO_2)_2$ and 2.5 mM [^{32}P]ATP at 6.2 Ci/mol], $CaCl_2$ (0.2 mM), EDTA (0.1 mM), mercaptoethanol (6.8 mM), and buffer (80 mM Hepes, pH 8.0). After 5 min 20 s, aliquots were removed for the determination of ^{32}P incorporation into protein. For determination of the residual activity with the tetradecapeptide, aliquots from the inactivation were initially diluted 10-fold with cold buffer (40 mM Hepes and 20 mM mercaptoethanol, pH 6.8) and then diluted 5-fold into an assay mixture containing, in final concentrations, kinase (4 μg/mL), tetradecapeptide (0.7 mM), MgATP [10 mM $Mg(CH_3CO_2)_2$ and 2.5 mM [^{32}P]ATP at 6.2 Ci/mol], $CaCl_2$ (0.2 mM), EDTA (0.1 mM), mercaptoethanol (4 mM), and buffer (80 mM Hepes, pH 8.0). After 5 min 20 s, aliquots were removed for determination of ^{32}P incorporation into protein. (○) Phosphorylase *b*; (△) tetradecapeptide. (B) Inactivation in the presence of Ca^{2+} and Mg^{2+}. Nonactivated phosphorylase kinase (0.18 mg/mL) was inactivated at 30 °C by oATP (10 μM) in Hepes buffer (27 mM, pH 6.8) in the presence of $CaCl_2$ (0.4 mM), $Mg(CH_3CO_2)_2$ (10 mM), EGTA (0.15 mM), and sucrose (0.7%). The residual activity was determined with phosphorylase *b* and tetradecapeptide as described in (A). (●) Phosphorylase *b*; (▲) tetradecapeptide.

modification was carried out in the absence of Ca^{2+} and Mg^{2+} ions (Figure 3A).

In preliminary experiments designed to measure the rate of inactivation of autophosphorylation caused by incubation of the enzyme with oATP in the presence of Ca^{2+} and Mg^{2+}, an initial activation was observed (data not shown). Fearing that the observed activation may have been due to autophosphorylation with oATP, we decided that oAMPPNP would be a better inhibitor to use for studies on the effect of the dialdehyde derivative in the presence of metals on autophosphorylation. However, when inactivation with oAMPPNP was carried out in the presence of Ca^{2+} and Mg^{2+} (Figure 3B), results similar to those with oATP were obtained. During the first 6 min of reaction with oAMPPNP, a slight activation seemed to occur when measured by autophosphorylation. Recall that a similar activation was observed with phosphorylase as substrate following incubation of the enzyme with oATP at pH 8.2 in the presence of Ca^{2+} and Mg^{2+} (King & Carlson, 1981). Extrapolation of the values in Figure 3B measured with phosphorylase also suggests that activation occurred with this substrate but appeared to be much slower than inactivation.

Although both the phosphorylase converting and autophosphorylating activities were inactivated at the same rate by oATP in the absence of metals (Figure 3A), the two activities decreased at dramatically different rates when the inactivation by oAMPPNP was carried out in the presence of Ca^{2+} and Mg^{2+}. After 4 min of inactivation with 10 μM oAMPPNP, there was virtually no change in the autophosphorylation activity whereas the ability to convert phos-

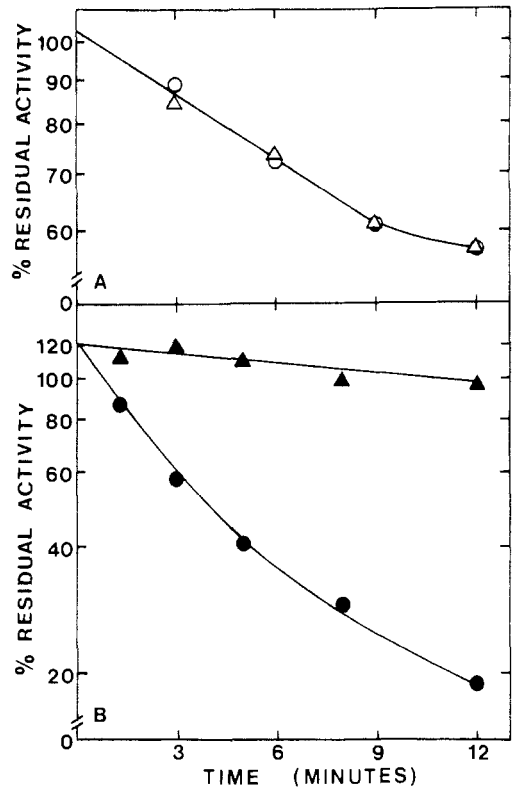

FIGURE 3: Comparison of the effect of inhibitor on autophosphorylation and the phosphorylation of phosphorylase *b*. (A) Inactivation in the absence of Ca^{2+} and Mg^{2+}. Nonactivated phosphorylase kinase (0.85 mg/mL) was inactivated at 30 °C with oATP (0.1 mM) in Hepes buffer (36 mM, pH 6.8) in the presence of EGTA (0.03 mM) and EDTA (0.08 mM). For determination of the residual activity toward autophosphorylation at the indicated times, aliquots were diluted 3-fold into an assay mixture containing, in final concentrations, kinase (0.28 mg/mL), MgATP [10 mM $Mg(CH_3CO_2)_2$ and 1.5 mM [^{32}P]ATP at 5.3 Ci/mol], $CaCl_2$ (0.6 mM), mercaptoethanol (19 mM), oATP (33 μM), and buffer (73 mM Hepes, pH 7.85). After 2 min at 30 °C, aliquots were removed for the determination of ^{32}P incorporation into protein. For determination of the residual activity with phosphorylase *b*, aliquots of the modified kinase were first diluted 1150-fold with cold buffer (40 mM Hepes and 20 mM mercaptoethanol, pH 6.8). These samples were then diluted 3-fold into an assay mixture containing, in final concentrations, kinase (0.25 μg/mL), phosphorylase *b* (4.4 mg/mL), MgATP [10 mM $Mg(CH_3CO_2)_2$ and 1.5 mM [^{32}P]ATP at 5.3 Ci/mol], $CaCl_2$ (0.6 mM), mercaptoethanol (23 mM), and buffer (75 mM Hepes, pH 7.85). After 5 min at 30 °C, aliquots were removed for determination of ^{32}P incorporation into protein. (○) Phosphorylase *b*; (△) autophosphorylation. (B) Inactivation in the presence of Ca^{2+} and Mg^{2+}. Nonactivated phosphorylase kinase (0.6 mg/mL) was inactivated at 30 °C with oAMPPNP (10 μM) in Hepes buffer (32 mM, pH 6.8) in the presence of EGTA (0.15 mM), EDTA (0.1 mM), sucrose (5.4%), $CaCl_2$ (0.4 mM), and $Mg(CH_3CO_2)_2$ (10 mM). For determination of the residual activity of autophosphorylation at the indicated times, aliquots were diluted 3-fold into an assay mixture containing, in final concentrations, kinase (0.2 mg/mL), MgATP [10 mM $Mg(CH_3CO_2)_2$ and 1.5 mM [^{32}P]ATP at 25.1 Ci/mol], $CaCl_2$ (0.25 mM), mercaptoethanol (15 mM), oAMPPNP (3.3 μM), and buffer (83 mM Hepes, pH 8.0). After 1.5 min at 30 °C, aliquots were removed for the determination of ^{32}P incorporation into protein. For determination of the residual activity by phosphorylase conversion, aliquots of the modified kinase were first diluted 200-fold with cold buffer (40 mM Hepes and 20 mM mercaptoethanol, pH 6.8). Aliquots of the initial dilution were then diluted 6-fold into an assay mixture containing, in final concentrations, kinase (0.5 μg/mL), phosphorylase *b* (4 mg/mL), MgATP [10 mM $Mg(CH_3CO_2)_2$ and 1.5 mM [^{32}P]ATP at 11.6 Ci/mol], $CaCl_2$ (0.25 mM), mercaptoethanol (15 mM), oAMPPNP (8 nM), and buffer (83 mM Hepes, pH 8.0). After 2 min at 30 °C, aliquots were removed for the determination of ^{32}P incorporation into protein. (●) Phosphorylase *b* conversion; (▲) autophosphorylation.

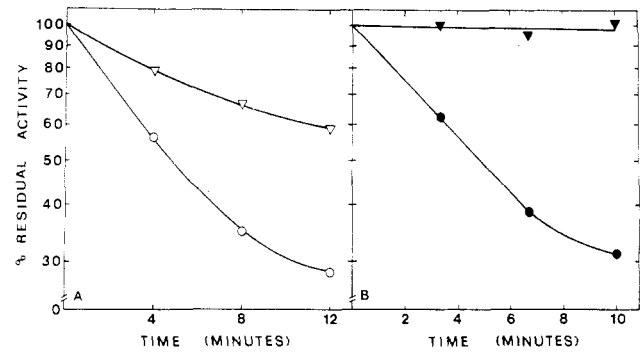

FIGURE 4: Comparison of the effect of oATP on the phosphorylation of phosphorylase *b* and troponin I. (A) Inactivation in the absence of Ca^{2+} and Mg^{2+}. Nonactivated phosphorylase kinase (0.5 mg/mL) was inactivated at 30 °C by oATP (75 μM) in Hepes buffer (34 mM, pH 6.8) in the presence of EDTA (40 μM) and sucrose (2%). For determination of the residual activity by phosphorylation of troponin I, aliquots were first diluted 10-fold with cold buffer (40 mM Hepes, 20 mM mercaptoethanol, pH 6.8) and then diluted an additional 5-fold into an assay mixture containing, in final concentrations, kinase (10 μg/mL), troponin I (0.2 mg/mL), MgATP [10 mM $Mg(CH_3CO_2)_2$ and 1.5 mM [^{32}P]ATP at 37 Ci/mol], $CaCl_2$ (0.25 mM), KCl (40 mM), EDTA (0.15 mM), mercaptoethanol (3.6 mM), oATP (1.5 μM), and buffer (70 mM Hepes, pH 8.0). After 3 min at 30 °C, aliquots were removed for determination of ^{32}P incorporation into protein. Control tubes were assayed in the absence of troponin I, and the small contribution due to autophosphorylation was subtracted from the results obtained in the presence of troponin I. For determination of the residual activity by phosphorylation of phosphorylase *b*, aliquots of the modified kinase were first diluted 200-fold with cold buffer (40 mM Hepes and 20 mM mercaptoethanol, pH 6.8) and then diluted an additional 5-fold into an assay mixture containing, in final concentrations, kinase (0.5 μg/mL), phosphorylase *b* (4 mg/mL), MgATP [10 mM $Mg(CH_3CO_2)_2$ and 1.5 mM [^{32}P]ATP at 17.6 Ci/mol], $CaCl_2$ (0.25 mM), KCl (40 mM), EDTA (0.15 mM), mercaptoethanol (6.1 mM), oATP (75 nM), and buffer (74 mM Hepes, pH 8.0). After 4 min at 30 °C, aliquots were removed for the determination of ^{32}P incorporation into protein. (▽) Troponin I; (○) phosphorylase *b*. (B) Inactivation in the presence of Ca^{2+} and Mg^{2+}. Nonactivated phosphorylase kinase (0.18 mg/mL) was inactivated at 30 °C by oATP (10 μM) in Hepes buffer (25 mM, pH 6.8) in the presence of EGTA (0.15 mM), $CaCl_2$ (0.4 mM), and $Mg(CH_3CO_2)_2$ (10 mM). For the determination of the residual activity with troponin I, aliquots were diluted 6-fold into an assay mixture containing, in final concentrations, kinase (30 μg/mL), troponin I (0.2 mg/mL), MgATP [10 mM $Mg(CH_3CO_2)_2$ and 1.5 mM [^{32}P]ATP at 28.2 Ci/mol], $CaCl_2$ (0.35 mM), KCl (40 mM), EDTA (0.2 mM), mercaptoethanol (14.9 mM), oATP (1.7 μM), and buffer (70 mM Hepes, pH 8.0). After 5 min at 30 °C, aliquots were removed for the determination of ^{32}P incorporation into protein. Control tubes were assayed in the absence of troponin I, and the small contribution due to autophosphorylation was subtracted from the results obtained in the presence of troponin I. For determination of the residual activity with phosphorylase *b*, aliquots of the modified kinase were initially diluted 100-fold with cold buffer (40 mM Hepes and 20 mM mercaptoethanol, pH 6.8) and then diluted 6-fold into an assay mixture containing, in final concentrations, kinase (0.3 μg/mL), phosphorylase *b* (4 mg/mL), MgATP [10 mM $Mg(CH_3CO_2)_2$; 1.5 mM [^{32}P]ATP at 28.2 Ci/mol], $CaCl_2$ (0.35 mM), KCl (40 mM), EDTA (0.2 mM), mercaptoethanol (5.3 mM), oATP (17 nM), and buffer (70.8 mM Hepes, pH 8.0). After 2 min at 30 °C, aliquots were removed for the determination of ^{32}P incorporation into protein. (▼) Troponin I; (●) Phosphorylase *b*.

phorylase had decreased by 60% (Figure 3B). The differentiation in the presence of metals of separate rates of inactivation with the two substrates is qualitatively the same as that observed with the tetradecapeptide and phosphorylase (Figure 2B).

Comparison of the Effect of oATP on the Phosphorylation of Troponin I and Phosphorylase b. When inactivation of phosphorylase kinase by oATP was measured by phosphorylation of troponin I and phosphorylase *b*, the rates of inactivation under identical modification conditions were different

for the two protein substrates. Figure 4A shows the results obtained with troponin I and phosphorylase *b* after inactivation of phosphorylase kinase by 75 μM oATP in the absence of Ca^{2+} and Mg^{2+}. The rate of inactivation measured with phosphorylase *b* is more than 2.5 times faster than that measured with troponin I. The results in Figure 4B show an even greater difference between the rates of inactivation. In the presence of Ca^{2+} and Mg^{2+}, 50% inhibition of phosphorylase kinase by 10 μM oATP in 5 min is observed with phosphorylase *b* while almost no inhibition is seen with troponin I as the substrate. The results in these experiments suggest that troponin I and phosphorylase *b* may be phosphorylated at two different types of catalytic sites on phosphorylase kinase.

Control experiments have ruled out that the difference in inactivation rates is due to the difference in workup of phosphorylase kinase for the two substrates after the modification reaction. Because phosphorylase *b* is a much better substrate than troponin I, the modified kinase was usually diluted 100-fold more for the assays with phosphorylase *b* than for the assays with troponin I. To repeat the above experiments without the dilution step, we raised the concentration of phosphorylase *b* in the assay to 10 mg/mL and lowered the assay time to 1 min. Furthermore, the concentration of kinase was lowered for troponin I and was now the same for troponin I and phosphorylase *b* conversion. These conditions showed no different results (data not shown) than were observed in Figure 4; however, this procedure was not routinely used because the concentrations of substrate and kinase as well as the assay times were impractical.

Comparison of the Effect of oATP on the Phosphorylation of Troponin T and Phosphorylase b. The results of the inactivation of activated phosphorylase kinase by 10 μM oATP in the presence of Ca^{2+} and Mg^{2+} as measured by the phosphorylation of troponin T and phosphorylase *b* are shown in Figure 5. The inactivation rate was 10 times faster when the residual activity of phosphorylase kinase was measured by phosphorylase *b* conversion than when measured by phosphorylation of troponin T.

Phosphorylated phosphorylase kinase was used in this experiment in order to avoid the large amount of ^{32}P incorporation by autophosphorylation that otherwise would have been observed in the assays of residual activity measured with troponin T as substrate. Troponin T was the most slowly phosphorylated substrate used in this work. In separate experiments, it was found that because of the high concentrations of nonactivated kinase required in the assays, the contribution due to autophosphorylation accounted for as much as 20% of the total ^{32}P incorporation into protein during assays for residual activity. We did not feel justified in simply subtracting out such a large contribution, and in addition, we were concerned about potential autoactivation resulting from the autophosphorylation. To avoid these problems, we employed enzyme that had already been autophosphorylated. Despite our concerns, however, the same final results were obtained when nonactivated enzyme was used (data not shown).

The inset of Figure 5 shows the results obtained when the inactivation was carried out in the absence of metals. Once again a differential rate of inactivation was observed. The inactivation rate measured by phosphorylase *b* conversion was greater than 10 times faster than that measured by phosphorylation of troponin T. In this experiment we were forced to use nonphosphorylated enzyme because, as was shown in the previous paper, the phosphorylated form shows no significant inactivation by oATP in the absence of Ca^{2+} and Mg^{2+}

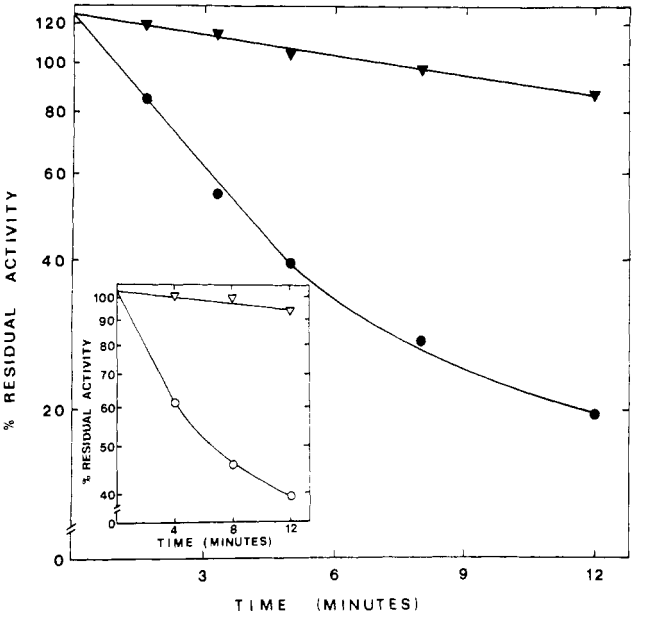

FIGURE 5: Comparison of the effect of oATP on the phosphorylation of troponin T and phosphorylase *b*. Inactivation in the presence of Ca^{2+} and Mg^{2+}. Phosphorylated phosphorylase kinase (0.12 mg/mL) was inactivated at 30 °C by oATP (10 μM) in Hepes buffer (30 mM, pH 6.8) in the presence of EGTA (0.15 mM), EDTA (44 μM), sucrose (2.2%), $CaCl_2$ (0.4 mM), and $Mg(CH_3CO_2)_2$ (10 mM). For determination of the residual activity at the indicated times with troponin T, aliquots were diluted 6-fold into an assay mixture containing, in final concentrations, kinase (20 μg/mL), troponin T (0.3 mg/mL), MgATP [10 mM $Mg(CH_3CO_2)_2$ and 1.5 mM [^{32}P]ATP at 31.7 Ci/mol], $CaCl_2$ (0.25 mM), KCl (75 mM), EDTA (0.15 mM), mercaptoethanol (10 mM), oATP (1.7 μM), and buffer (73 mM Hepes, pH 8.0). After 3 min at 30 °C, aliquots were removed for the determination of ^{32}P incorporation into protein. For determination of the residual activity with phosphorylase *b* as the substrate, aliquots of the modified kinase were first diluted 60-fold with cold buffer (40 mM Hepes and 20 mM mercaptoethanol, pH 6.8) and then diluted an additional 6-fold into an assay mixture containing, in final concentrations, kinase (0.33 μg/mL), phosphorylase *b* (4 mg/mL), MgATP [10 mM Mg $(CH_3CO_2)_2$; 1.5 mM [^{32}P]ATP at 17 Ci/mol], $CaCl_2$ (0.25 mM), KCl (75 mM), EDTA (0.15 mM), mercaptoethanol (10 mM), and buffer (73 mM Hepes, pH 8.0). After 3 min at 30 °C, aliquots were removed for the determination of ^{32}P incorporation into protein. (▼) Troponin T; (●) phosphorylase *b*. (Inset) Inactivation in the absence of Ca^{2+} and Mg^{2+}. Nonactivated phosphorylase kinase (0.15 mg/mL) was inactivated at 30 °C by oATP (50 μM) in Hepes buffer (32 mM, pH 6,8) in the presence of EDTA (0.012 mM). For determination of residual activity at the indicated times with troponin T as the substrate, aliquots were diluted 5-fold into an assay mixture containing, in final concentrations, kinase (30 μg/mL), troponin T (0.3 mg/mL), MgATP [10 mM $Mg(CH_3CO_2)_2$ and 1.5 mM [^{32}P]ATP at 25.9 Ci/mol], $CaCl_2$ (0.25 mM), KCl (75 mM), EDTA (0.15 mM), mercaptoethanol (10 mM), oATP (10 μM), and buffer (68.8 mM Hepes, pH 8.0). After 5 min at 30 °C, aliquots were removed for the determination of ^{32}P incorporation into protein. Control tubes were assayed in the absence of troponin T, and the contribution due to autophosphorylation was subtracted from the results obtained in the presence of troponin T. For determination of the residual activity with phosphorylase *b* as the substrate, the modified kinase was first diluted 100-fold with cold buffer (40 mM Hepes and 20 mM mercaptoethanol, pH 6.8). These samples were then diluted an additional 6-fold into an assay mixture containing, in final concentrations, kinase (0.25 μg/mL), phosphorylase *b* (4 mg/mL), MgATP [10 mM $Mg(CH_3CO_2)_2$ and 1.5 mM [^{32}P]ATP at 10.9 Ci/mol], $CaCl_2$ (0.25 mM), KCl (75 mM), EDTA (0.15 mM), mercaptoethanol (5.6 mM), and buffer (69.3 mM Hepes, pH 8.0). After 5 min at 30 °C, aliquots were removed for the determination of ^{32}P incorporation into protein. (▽) Troponin T; (○) phosphorylase *b*.

(King & Carlson, 1981). The results in this experiment are again consistent with the participation of two different types of catalytic sites of phosphorylase kinase in the phosphorylation of troponin T and phosphorylase *b*.

Discussion

In the preceding paper, the dialdehyde derivative of ATP was characterized as being a specific and valid affinity label of the adenine nucleotide binding site in phosphorylase kinase responsible for phosphorylase conversion (King & Carlson, 1981). This finding allowed us to employ differential inactivation methods in order to investigate whether one or two types of catalytic sites exist on phosphorylase kinase. We measured the rate of inactivation by oATP with various substrates of phosphorylase kinase and compared that rate with the rate of inactivation observed with phosphorylase *b* as substrate. If only one type of catalytic site was used to phosphorylate both substrates, then only a single rate of inactivation should be observed with those substrates. On the other hand, if two different types of catalytic sites were required and if those sites bound, or reacted with, the affinity label differently, then one would expect to observe different rates of inactivation with the two substrates.

Upon comparing the rate of inactivation observed with five different substrates against that measured with phosphorylase, three different classes of substrates were observed. In the first class, of which glycogen synthase was the sole member, only one rate of inactivation was observed for phosphorylase and the comparison substrate. It mattered not whether the inactivation was carried out in the presence or absence of Ca^{2+} and Mg^{2+}. For the second class of substrates, of which both troponins were members, the rate of inactivation with phosphorylase was much faster than that with the comparison substrates regardless of whether the modification was carried out with or without metals. The third class of substrates, composed of the tetradecapeptide and phosphorylase kinase itself, showed parallel rates of inactivation with phosphorylase when the modification was performed in the absence of Ca^{2+} and Mg^{2+}. However, when the modification was performed in the presence of Ca^{2+} and Mg^{2+}, the inactivation measured with phosphorylase proceeded much faster than that measured with the comparison substrates. Each of these three classes of substrates will be discussed separately.

The findings with glycogen synthase were not surprising and, in fact, verified observations made by others with this substrate. Upon observing similar changes in phosphorylation of glycogen synthase and phosphorylase *b* by phosphorylase kinase following variation of pH, Ca^{2+} concentration, and the activation state of the kinase, DePaoli-Roach et al. (1979) postulated that the same catalytic center was involved in the phosphorylation of both substrates. This hypothesis was strengthened by Soderling et al. (1979), who found that antiserum to phosphorylase kinase inhibited the phosphorylation of glycogen synthase in parallel with phosphorylase *b*. Furthermore, they observed that phosphorylase *b* inhibited conversion of glycogen synthase by phosphorylase kinase. Our studies on inactivation by the dialdehyde affinity label can also be interpreted to mean that glycogen synthase and phosphorylase *b* are phosphorylated by the same type of catalytic center in phosphorylase kinase. This finding not only verifies previous observations but also acts to validate the use of oATP for differential inactivation studies of the type presented in this report. Considering that glycogen synthase and phosphorylase are large proteins phosphorylated by phosphorylase kinase at homologous regions of their amino termini (Soderling et al., 1979), it is not surprising that both substrates would be phosphorylated at the same catalytic center on the kinase.

The most straightforward explanation for the behavior of phosphorylase kinase toward the second class of substrates, represented by the troponins, is that they are phosphorylated at a different catalytic site than that utilized for conversion of phosphorylase. The data obtained in this study (Figures 4 and 5) could be explained by the preferred reaction of oATP with the phosphorylase converting site, as opposed to the site for troponin phosphorylation. Such an interpretation is consistent with the findings of Dickneite et al. (1978), who proposed that troponin T and phosphorylase are phosphorylated by two different catalytic centers in phosphorylase kinase. Their hypothesis was based upon differential inhibition of phosphorylation of the two substrates by antibodies against phosphorylase kinase as well as the inability of troponin T to inhibit phosphorylase conversion by phosphorylase kinase. The unexplained findings of Stull et al. (1972) that phosphorylation of troponin I by phosphorylase kinase was refractory to the activation state of the enzyme and that the ratio of activity at pH 6.8 to that at pH 8.2 was 0.7 could also be rationalized if phosphorylase *b* and troponin I were phosphorylated at different catalytic centers in phosphorylase kinase.

The data obtained with the third class of substrates (phosphorylase kinase itself and the tetradecapeptide), for which parallel inactivation with phosphorylase was observed in the absence of metals and different rates of inactivation in the presence of Ca^{2+} and Mg^{2+}, are much more difficult to interpret. In part, the difficulty in understanding these substrates is that the molecular mechanism through which Ca^{2+} and Mg^{2+} synergistically influence phosphorylase kinase, including its inactivation by oATP, has not been elucidated. Another serious problem in interpreting the results with autophosphorylation is that phosphorylase kinase served both as the modified enzyme and as the substrate in those experiments. It is not known whether prior incubation with oATP, or Ca^{2+} plus Mg^{2+}, changes the ability of phosphorylase kinase to act as its own substrate. Furthermore, if two catalytic centers do exist on phosphorylase kinase, it is not unreasonable to assume, because of multiple phosphorylations of both the α and β subunits (Carlson et al., 1979), that both could catalyze autophosphorylation but at different rates and with different specificities. This possibility that two different catalytic sites might not have an all-or-none specificity for substrate also could help explain some of the paradoxical data that have been previously obtained with peptide substrates of phosphorylase kinase. For instance, the tetradecapeptide is a good substrate for both cyclic AMP dependent protein kinase and phosphorylase kinase whereas phosphorylase *b* is not converted by cyclic AMP dependent protein kinase (Tessmer et al., 1977). Perhaps the peptide is not wholly phosphorylated at the same catalytic center utilized for phosphorylase conversion. Along this line, it has been noted (Embi et al., 1979) that even though glycogen synthase and phosphorylase are phosphorylated at rather similar rates, glycogen synthase does not contain an arginine residue two residues on the C-terminal side of the convertible serine. Yet, studies with homologous peptides have shown this particular arginine to be critical in the interaction of phosphorylase kinase with the peptides (Tessmer et al., 1977; Viriya & Graves, 1979). We can only conclude that the results obtained with the peptide and autophosphorylation neither support nor dispute the results obtained with glycogen synthase, troponin I, and troponin T but that due to the complexity of the system their interpretation is not possible from the data at hand.

We believe that the sum of our data and supporting evidence in the literature are most easily explained by a model that proposes two types of catalytic sites on phosphorylase kinase which show different, but not absolute, specificities for different substrates. The two types of catalytic sites, in turn, are most

easily visualized as being initially heterogeneous. For instance, troponin could perhaps be preferentially phosphorylated by one subunit and phosphorylase and glycogen synthase by another. Alternatively, all catalytic sites could initially be physically and catalytically homogeneous but become catalytically heterogeneous after reacting with the oATP. This could occur, for instance, if a modified site was still capable of phosphorylating certain substrates (the troponins), but not others (phosphorylase and glycogen synthase). Yet another explanation for our data could be the binding of the oATP affinity label to a regulatory site, resulting in conformational changes at a single type of catalytic site which are then expressed differently with different protein substrates. Experiments currently under way in our laboratory with other adenine nucleotide affinity labels capable of forming stable, isolable covalent linkages should help to distinguish the correct mechanism from among these various possibilities.

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Interspersion of Mouse Satellite Deoxyribonucleic Acid Sequences†

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ABSTRACT: DNA sequences with homology to the major (A + T)-rich mouse satellite component were localized in CsCl gradients by hybridization with a labeled satellite cRNA probe. Although, as expected, most of the hybridization was to DNA in the satellite-rich shoulder, substantial radioactive cRNA hybridized with DNA from denser regions of the gradient. Further examination revealed that hybridization to main-band DNA was not due to physical trapping of satellite DNA in the gradient, and melting experiments argue that the associated radioactivity was due to true RNA/DNA hybridization. Nearest-neighbor analysis of hybridized [α-^{32}P]CTP-labeled l-strand cRNA indicates that hybridization to main-band DNA is by the satellite cRNA and not a contaminant. Together, these data argue that mouse satellite-like sequences are interspersed within the main-band fraction of DNA. For the support of this contention, total mouse DNA, purified main-band DNA, and purified satellite DNA were digested with *Eco*RI, sedimented in a sucrose gradient, and hybridized with labeled satellite cRNA. Mouse satellite DNA is not cleaved with *Eco*RI, so that purified *Eco*RI-digested satellite DNA sediments as a high molecular weight component. When total mouse DNA is digested with *Eco*RI, the majority of satellite-like sequences remain as high molecular weight DNA; however, significant amounts of satellite-like sequences sediment with the bulk of the lower molecular weight digested DNA, lending further credence to the argument that satellite-like sequences are interspersed with main-band DNA.

Nuclear satellite DNAs are characteristic of most, if not all, higher eukaryotes [for review, see Skinner (1977) and John & Miklos (1979)]. These DNAs are comprised of highly repeated nucleotide sequences ordered in a tandem array. They vary in complexity, ranging from the very simple sequences comprising the crab poly[d(A-T)] satellite (Sueoka & Cheng, 1962) and the heptanucleotide repeats found in *Drosophila virilis* (Gall & Atherton, 1974) to components with a periodicity as great as 1400 base pairs (bp) such as calf

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