The calcium-dependent regulator (CDR), which activates several enzymes, stimulated nonactivated phosphorylase kinase 2- to 8-fold at pH 6.8 and to a much smaller extent at pH 8.2. Half-maximal stimulation occurred with 15 nM CDR. The stimulation by CDR was independent of the concentration of phosphorylase kinase and was observed when the substrate was phosphorylase b, glycogen synthase, or phosphorylase kinase itself. The addition of CDR did not affect the activation constant for glycogen or the apparent $K_m$ values for phosphorylase b and ATP. CDR had little or no effect on phosphorylase kinase which had been activated by phosphorylation. In contrast to CDR, glycogen increased phosphorylase kinase activity 4- to 8-fold even at pH 8.2 and after activation by phosphorylation.

In the absence of added CDR, the activity of nonactivated and activated phosphorylase kinase was reversibly inhibited by trifluoperazine, a phenothiazine derivative which interacts with CDR. In addition, 80 $\mu$M trifluoperazine eliminated the stimulation by added CDR when the drug was added either before or after the kinase had been incubated for 10 min with CDR in the phosphorylase kinase reaction mixture.

When the enzyme was "activated" by trypsin treatment, the response to both glycogen and CDR was lost. As a result, trypsin treatment caused activation when activity was measured in the absence of glycogen and CDR but inactivation when activity was measured in the presence of glycogen and CDR. Trypsin treatment also rendered the enzyme less sensitive to inhibition by EGTA, a calcium chelator which completely inhibits nonproteinolyzed phosphorylase kinase.

The results presented here suggest that the activity of phosphorylase kinase is dependent on the presence of CDR as a subunit, which can be inhibited by trifluoperazine, and that the effect of added CDR is dependent on interaction at a second binding site.

Phosphorylase kinase catalyzes the calcium-dependent phosphorylation of phosphorylase $b$ (1) and glycogen synthase (2-4). It can also catalyze its own phosphorylation in an intermolecular process known as autophosphorylation (5). Phosphorylase kinase functions to mobilize glycogen in response to hormonal and neuronal control (6) and may play a role in the regulation of muscle contraction via the phosphorylation of tropocin (7). Phosphorylase kinase activity is affected by covalent modifications resulting from autophosphorylation, from phosphorylation by cyclic AMP-dependent protein kinase, and from limited proteolysis (8). These covalent modifications enhance activity to a greater extent at pH 6.8 than at pH 8.2. The catalytic activity of purified phosphorylase kinase is also influenced in a noncovalent fashion by metabolic effectors such as glycogen (1). Glycogen stimulates the rate of autophosphorylation (9), the rate of phosphorylase $b$ phosphorylation (1), and to a smaller extent, glycogen synthase phosphorylation (10).

The activity of native phosphorylase kinase is completely dependent on calcium (11). The calcium dependence of enzymes such as cyclic nucleotide phosphodiesterase (12, 13), myosin light chain kinase (14), adenylate cyclase (15), and (Ca$^{2+}$-Mg$^{2+}$)-ATPase (16, 17) is conferred by a heat-stable calcium-dependent regulator (CDR) protein which binds calcium (18). Cohen et al. (19, 20) presented evidence that 4 mol of CDR are present in the purified phosphorylase kinase tetramer and suggested that CDR is the subunit which mediates the calcium dependency of this enzyme. However, the binding of CDR to phosphorylase kinase differs from the binding to cyclic nucleotide phosphodiesterase (12, 13) and myosin light chain kinase (14) in that CDR readily dissociates from the latter enzymes during purification in the absence of calcium, whereas phosphorylase kinase purified under similar conditions retains CDR (19, 20). Despite the presence of nondissociable CDR in purified phosphorylase kinase, the addition of CDR from bovine brain increases enzyme activity (19-21). CDR also stimulates phosphorylase kinase activity which is present as a contaminant in preparations of glycogen synthase (4).

Phenothiazine antipsychotic agents such as trifluoperazine have been used as a tool to study the mechanism of action of CDR since they bind to this regulator (22) in the presence of calcium, preventing the stimulation of cyclic nucleotide phosphodiesterase (22), adenylate cyclase (23), and phosphorylase kinase (21) by added CDR. In this paper, we report the effects of CDR on the kinetic properties of phosphorylase kinase and describe the conditions needed to observe stimulation by CDR. We also show that trifluoperazine can inhibit phosphorylase kinase even in the absence of added CDR.

The abbreviations used are: cyclic AMP, adenosine 3',5'-monophosphate; EGTA, ethylene glycol bis[bisaminomethyl] ether) N,N',N; tetaacetic acid; CDR, calcium-dependent regulator.


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Stimulation of Phosphorylase Kinase by CDR

**EXPERIMENTAL PROCEDURES**

**Kinase Assays**—Unless otherwise indicated, phosphorylase kinase activity was measured at 30°C and at pH 6.8 in a mixture containing 1 mg of phosphorylase b/ml, 90 mM β-glycerophosphate, 50 mM Tris, 10 to 20 mM β-mercaptoethanol, 2 to 4% glycerol, 0.5 to 2 mM EDTA, 10 mM magnesium acetate, 20 μM [γ-32P]ATP (50 to 300 cpm/μmol). Where indicated, the reaction mixtures contained 0.2 mM CaCl₂, 1 mM EDTA, 6 to 16 μM of CDR/ml (0.4 to 0.6 μM), or 0.2 mM trifluoperazine. Although the concentration of ATP is below the Michaelis constant, i.e. a lag in the reaction rate (1,25), the progress of the reaction mixture for 15 to 20 min at 30°C. This incubation time varied from one experiment to another but did not affect stimulation by CDR. During the reaction, aliquots were removed to determine the quantity of protein-bound 32P by the filter paper method described previously (24). Because phosphorylase kinase sometimes exhibits hysteresis, i.e. a lag in the reaction rate (1,26), the progress of most reactions was monitored by removing aliquots at several time points after the addition of MgATP. Unless indicated otherwise, the reaction rate was constant for the duration of the incubation time. Reaction velocities were calculated by least squares analysis using the activities measured at several time points.

**Autoactivation of Phosphorylase Kinase**—Unless otherwise indicated, autophosphorylation reactions were carried out at 30°C at pH 8.2 in reaction mixtures which contained 40 to 100 μg of phosphorylase kinase/ml, 75 mM β-glycerophosphate, 62.5 mM Tris, 0.25 mM CaCl₂, 0.25 mM EDTA, 45 μM [γ-32P]ATP, and 12.5 mM magnesium acetate. Reaction of phosphorylase kinase by protein kinase was carried out at 30°C in reaction mixtures containing 90 to 170 μg of phosphorylase kinase/ml, 1 μg of catalytic subunit of the cyclic AMP-dependent protein kinase/ml, 25 to 33 μM [γ-32P]ATP, 12.5 to 15.7 mM magnesium acetate, 3.05 to 1.25 mM EDTA, 0.3 mM dithiothreitol, 75 to 100 mM Tris, and 75 to 100 mM β-glycerophosphate (pH 6.8). After 30 min, the reaction mixture was diluted 50-fold in cold 50 mM glycerophosphate (pH 7.0) which contained excess heat-stable inhibitor of the protein kinase (26) and 3.2 mM EDTA. Unless otherwise indicated, trypsin treatment of phosphorylase kinase was at 30°C in a reaction mixture containing 80 to 225 μg of phosphorylase kinase/ml, 8 to 13 μg of trypsin/ml, 90 to 100 mM β-glycerophosphate, 60 to 80 mM Tris, 1.3 mM EDTA, and 0.5 mM dithiothreitol at pH 6.8. The reactions were stopped after 30 min by diluting into cold 50 mM glycerophosphate buffer (pH 7.8) which contained excess soybean trypsin inhibitor.

**Enzyme Purifications**—Rabbit skeletal muscle phosphorylase kinase was purified according to the method of Cohen (27) through the Sepharose 4B column step. For some experiments, the kinase was chromatographed on a DEAE-cellulose column, but to the best of our knowledge, this further purification had no influence on the properties of phosphorylase kinase with a specific activity of 1000 pmol phosphorylase/min/μg. Additional evidence that phosphorylase kinase specifically binds CDR was recently reported by DePaoli-Roach et al. (36). They found that in the presence of calcium, phosphorylase kinase was bound to a CDR affinity column. Quantitative recovery of the enzyme was obtained by eluting the column with EGTA.

**Autophosphorylation of a Fresh Preparation of Phosphorylase Kinase**—An activity that was stimulated by CDR in the absence of added CDR should be dependent on the concentration of the enzyme. To test this possibility, the effect of CDR was measured when the kinase concentration was varied from 0.5 to 24 μg. The velocities were constant for the first 1% to 3 min of reaction and were directly dependent on kinase concentration whether CDR was present or not (Table I). The stimulation by CDR was also independent of the concentration of protein-bound 32P in the absence of added CDR of glycogen (results not shown). Furthermore, attempts to deplete phosphorylase kinase of CDR by gel filtration under several conditions were unsuccessful. These results indicate that CDR is not dissociating from the enzyme, and that the enzyme is stimulated by CDR which binds in excess of that found in the purified preparation. Additional evidence that phosphorylase kinase specifically binds CDR was recently reported by DePaoli-Roach et al. (36). They found that in the presence of calcium, phosphorylase kinase was bound to a CDR affinity column. Quantitative recovery of the enzyme was obtained by eluting the column with EGTA.

**Materials**—[γ-32P]ATP was either purchased from Amersharm or prepared by the method of Wellsh and Johnson (34). Glycogen (oyster type II) was obtained from Sigma and purified on a mixed bed ion exchange column (35). Trypsin (code: TRF PKC) and soybean trypsin inhibitor were obtained from the Worthington Biochemical Corp. Trifluoperazine was provided by Smith, Kline and French Pharmaceuticals.

**RESULTS**

**Effect of CDR on Nonactivated Phosphorylase Kinase**—In the presence of calcium, CDR stimulated the rate at which nonactivated phosphorylase kinase catalyzed the phosphorylation of phosphorylase b (Fig. 1). The activation was 4-fold at pH 6.8 and minimal at pH 8.2. The net amount of CDR-dependent activity at pH 6.8 was about double that at pH 8.2.

**Effect of CDR on Kinetic Properties of Nonactivated Phosphorylase Kinase**—The effects of CDR on the kinetic properties of phosphorylase kinase were determined to see if CDR increased the sensitivity to glycogen, ATP, or phosphorylation. In a series of six experiments, the relative stimulation by CDR (+ CDR activity– CDR activity) was 3.50 ± 0.47 (mean ± S.E.) at pH 6.8 and 1.12 ± 0.06 at pH 8.2.

**Effect of CDR on Kinetic Properties of Nonactivated Phosphorylase Kinase**—The effects of CDR on the kinetic properties of phosphorylase kinase were determined to see if CDR increased the sensitivity to glycogen, ATP, or phosphorylation. In a series of six experiments, the relative stimulation by CDR was 1.78 ± 0.33 (mean ± S.E.) at pH 6.8 and 1.18 ± 0.11 at pH 8.2.

**Stimulation of Phosphorylase Kinase by CDR**

In a series of experiments, the relative stimulation by CDR was 1.78 ± 0.33 (mean ± S.E.) at pH 6.8 and 1.18 ± 0.11 at pH 8.2.
At pH 6.8, glycogen stimulated the activity of non-activated phosphorylase kinase about 1.6-fold when CDR was present (Table II). Krebs et al. (1) reported that the A0.5 for glycogen was 0.1 mg of glycogen/ml whether measured in the absence or presence of CDR (Table II). Krebs et al. (1) reported that the A0.5 for glycogen was 0.1 mg of glycogen/ml at pH 8.2, which is about 30-fold higher than described here. The difference could result from either differences in pH or in properties of the glycogen. At pH 8.2, glycogen also stimulated nonactivated kinase with or without CDR although the stimulation was less than at pH 6.8 (results not shown).

In the presence of 1.6 mg of glycogen/ml, nonactivated phosphorylase kinase had apparent Km values for phosphorylase b of 3 mg/ml in the presence or absence of CDR (Table II). Glycogen was utilized in this study because it lowers the Km for phosphorylase (1), allowing a more accurate measurement at pH 6.8.

CDR stimulated phosphorylase kinase at all concentrations of ATP tested. At pH 6.8, the apparent Km for ATP was 26 \(\mu\)M in the absence and 67 \(\mu\)M in the presence of CDR (Table II). At pH 8.2 in the presence of CDR, the apparent Km for ATP was found to be 220 \(\pm\) 70 \(\mu\)M (results not shown) in agreement with previous reports (1, 38). A consequence of the lower Km at pH 6.8 is that the pH 6.8:8.2 activity ratio will be elevated when the concentration of ATP is significantly below 200 \(\mu\)M.

Lags of 1 to 3 min followed by linear time courses were observed in some of the kinetic studies but hysteresis was independent of the concentration of ATP, phosphorylase, or phosphorylase kinase activity.

TABLE I

<table>
<thead>
<tr>
<th>Kinase concentration (nM)</th>
<th>32P incorporated (pmol/min/25 (\mu)l reaction mixture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-CDR</td>
<td>+CDR/CDR/CDR</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5 2.1 4.2</td>
</tr>
<tr>
<td>1.6</td>
<td>1.6 5.8 3.6</td>
</tr>
<tr>
<td>4.8</td>
<td>4.8 15.8 3.2</td>
</tr>
<tr>
<td>24.0</td>
<td>22.2 65.7 3.0</td>
</tr>
</tbody>
</table>

FIG. 1. Effect of CDR on phosphorylation of phosphorylase b. Phosphorylase b was incubated as described under "Experimental Procedures" with 3 \(\mu\)g of nonactivated phosphorylase kinase/ml. The pH of the reaction mixture was 6.8 (a, b) or 8.2 (c, d) and included calcium with (closed) or without (open) CDR. The ordinate axis displays 32P incorporation per 300 pmol of phosphorylase b.

Phosphorylase kinase activity was determined at pH 6.8 as described under "Experimental Procedures" except that the reaction mixture contained excess heat-stable inhibitor of the cyclic AMP-dependent protein kinase. The ordinate axis displays 32P incorporation into 3.6 pmol of phosphorylase kinase (1 pmol = 0.335 \(\mu\)g of \(\alpha\)6\(\delta\) subunit).

Fig. 2. The effect of CDR on the rate of autophosphorylation. Phosphorylase kinase was incubated at pH 6.8 in the autophosphorylation reaction mixture described under "Experimental Procedures," with (a) or without (c) CDR. The reaction mixture also contained excess heat-stable inhibitor of the cyclic AMP-dependent protein kinase. The ordinate axis displays 32P incorporation into 3.6 pmol of phosphorylase kinase (1 pmol = 0.335 \(\mu\)g of \(\alpha\)6\(\delta\) subunit).

Phosphorylase kinase activity was determined at pH 6.8 as described in the text except that glycogen, phosphorylase, or ATP concentrations were varied. The A0.5 for glycogen was determined with a kinase concentration of 0.4 \(\mu\)g/ml. The Km values for phosphorylase b and for ATP were determined in the presence of glycogen at kinase concentrations of 0.16 \(\mu\)g/ml and 0.06 \(\mu\)g/ml, respectively. Kinetic constants and standard errors were calculated by fitting the data directly to the Michaelis-Menten equation by nonlinear least squares analysis (39).

TABLE II

<table>
<thead>
<tr>
<th>Varied substance</th>
<th>CDR</th>
<th>Km ((\mu)M)</th>
<th>Vmax (pmol/min/25 (\mu)l)</th>
<th>V* (pmol/min/25 (\mu)l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen (mg/ml)</td>
<td>-</td>
<td>0.12 (\pm) 0.04</td>
<td>44 (\pm) 5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.07 (\pm) 0.01</td>
<td>79 (\pm) 5</td>
<td>18</td>
</tr>
<tr>
<td>Phosphorylase b</td>
<td>-</td>
<td>3.1 (\pm) 0.4</td>
<td>410 (\pm) 20</td>
<td></td>
</tr>
<tr>
<td>(mg/ml)</td>
<td>+</td>
<td>3.0 (\pm) 0.2</td>
<td>1100 (\pm) 40</td>
<td></td>
</tr>
<tr>
<td>ATP ((\mu)M)</td>
<td>-</td>
<td>26 (\pm) 7</td>
<td>40 (\pm) 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>67 (\pm) 6</td>
<td>250 (\pm) 8</td>
<td></td>
</tr>
</tbody>
</table>

*a When glycogen was varied, the data were fitted to the equation:

\[ V - V_0 = \frac{V_{\text{max}} \cdot [\text{glycogen}]}{A_{0.5} + [\text{glycogen}]} \]

where V\(_{\text{max}}\) represents the net increase in activity at saturating glycogen concentrations and V\(_0\) represents the activity observed in the absence of glycogen.
glycogen, or CDR. Velocities were calculated by least squares analysis on the linear part of the time course. Although the double reciprocal plots were linear, a strict interpretation of the $K_m$ is probably not appropriate. Nevertheless, it is clear that the degree of stimulation by CDR was independent of the concentration of glycogen, phosphorylase, or MgATP. Therefore, the stimulation by CDR is probably not due to an increased affinity of phosphorylase kinase for glycogen or either of its substrates, i.e. the effect of CDR is on the $V_{max}$.

**Effect of CDR on Phosphorylase Kinase Activated by Phosphorylation**—Phosphorylase kinase which had been phosphorylated to the extent of 0.6 mol/335,000 g of kinase by incubation with the catalytic subunit of the cyclic AMP-dependent protein kinase was stimulated about 25% by CDR when assayed at pH 6.8 (Fig. 4). There was no effect of CDR when assayed at pH 8.2. In most experiments the phosphorylated enzyme was completely insensitive to CDR at either pH.5

**Effect of Trifluoperazine on Phosphorylase Kinase Activity**—Cohen et al. (19) concluded that phosphorylase kinase contains CDR as a subunit, which is released upon boiling of the enzyme. In support of this conclusion, we found that the addition of boiled phosphorylase kinase increased the activity of nonactivated phosphorylase kinase at pH 6.8 to the same extent as bovine brain CDR (results not shown). Furthermore, we observed that trifluoperazine inhibited phosphorylase kinase in the absence of added CDR (Fig. 5). Presumably inhibition by this drug under these conditions results from binding to the high affinity, calcium-dependent binding sites on the CDR subunit (22), since the inhibition was the same at 10 to 160 µM trifluoperazine. In this and other experiments at pH 6.8, trifluoperazine inhibited nonactivated kinase 27 to 83%. Similar inhibition was seen at pH 8.2, for the autophosphorylation reaction (results not shown), and with activated enzyme (see below). The reversibility of the inhibition by trifluoperazine was tested in a separate experiment (not shown) by incubating nonactivated kinase with 10 mM MgCl$_2$ and 0.2 mM CaCl$_2$ for 2 h with or without 0.1 mM trifluoperazine. Prior to assay at pH 8.2, the enzyme was diluted to lower the concentration of trifluoperazine to 0.4 µM. The decrease in enzyme activity during the incubation was 3% in the absence and 5% in the presence of trifluoperazine indicating that trifluoperazine did not inhibit the enzyme irreversibly.

The activity seen in the presence of concentrations of trifluoperazine which caused maximal inhibition was still dependent on the presence of calcium, since this activity was completely inhibited by EGTA (results not shown). This suggests that some of the CDR in nonactivated phosphorylase kinase is bound in such a manner that it is not susceptible to inhibition by trifluoperazine, or that calcium acts at other subunits. Phosphorylase kinase apparently contains more than one type of calcium binding site (11, 40). In addition to partially inhibiting phosphorylase kinase activity in the absence of CDR, 80 µM trifluoperazine completely prevented the effect of added CDR (Fig. 5). Since activated phosphorylase kinase is markedly inhibited by trifluoperazine in the absence of added CDR, and since added CDR does not stimulate the activated enzyme it appears that the CDR subunit is essential for full activity, whereas added CDR is not.

It seemed possible that the stimulation by added CDR could involve activation by covalent modification of the enzyme, e.g. phosphorylation or proteolysis. If this were the mechanism by which CDR stimulated phosphorylase kinase, then trifluoperazine would not inhibit if it were added during the course of a phosphorylase kinase reaction, i.e. after the putative activation had occurred. However, Fig. 6 shows that the effect of CDR was completely reversed when trifluoperazine was added to a kinase reaction mixture 10 min after the reaction had been initiated, suggesting direct and reversible stimulation of the kinase by CDR. However, we cannot definitively rule out an indirect effect of CDR via increased autophosphorylation since trifluoperazine also inhibited autophosphorylation in the absence of added CDR (Fig. 5).

**Effect of CDR, Trifluoperazine, and EGTA on Trypsin-treated Phosphorylase Kinase**—When phosphorylase kinase (225 µg/ml) was incubated with trypsin (13 µg/ml) for 30 min at 30°C, the activity at pH 6.8 increased 4-fold and the pH 6.8:8.2 activity ratio increased from 0.15 to 0.61. At either pH, the trypsin-treated kinase showed the same rate of phosphorylase b phosphorylation whether it was assayed in the presence of EGTA, calcium, or calcium and CDR. This lack of response to CDR is in agreement with recent findings of DePaoli-Roach et al. (36). An interesting aspect of trypsin treatment is that phosphorylase kinase becomes catalytically active toward phosphorylase b and glycogen synthase in the presence of EGTA (Ref. 10 and Fig. 7). In the presence of 1 mM EGTA and no added calcium, nonactivated phosphorylase kinase was virtually inactive toward phosphorylase b (results not shown), but trypsin-treated phosphorylase kinase was fully or partially active.
Trifluoperazine inhibited kinase activity even in the presence of EGTA (Fig. 7). Although the binding of trifluoperazine to CDR normally requires calcium (22), it is possible that trypsin treatment of phosphorylase kinase converts the CDR subunit to a form which is calcium-independent, but trifluoperazine-sensitive. Alternatively, either some of the inhibition by trifluoperazine may be independent of CDR or trypsin-treated kinase may bind calcium even in the presence of EGTA.

**Effect of Glycogen on Phosphorylase Kinase**—When phosphorylase kinase was activated by phosphorylation in the presence of catalytic subunit of the cyclic AMP-dependent protein kinase, the response to glycogen persisted (Table III) whereas the response to CDR was greatly decreased (Fig. 4). Similar results (not shown) were obtained with autophosphorylation. On a percentage basis the stimulation by glycogen was greater at pH 6.8 than at pH 8.2 for either nonactivated (Table III and Ref. 1) or phosphorylated phosphorylase kinase (Table III). Glycogen has a minimal effect when the substrate is glycogen synthase (10) or the synthetic NH₂-

![Graph](image)

**Fig. 6. Effect of adding trifluoperazine during the phosphorylase kinase reaction.** Phosphorylase b was incubated at pH 6.8 as described under "Experimental Procedures" with 0.1 μg of nonactivated (○, ◦) or auto-activated (□) phosphorylase kinase/ml in a reaction mixture containing 2 mg of glycogen/ml and calcium with (closed symbols) or without (open symbols) CDR. After 10 min of incubation, 2.5 μl of H₂O (broken lines) or 10 mM trifluoperazine (solid lines) were added to 125 μl of reaction mixture. The ordinate axis displays 32P incorporated into 250 pmol of phosphorylase b.

![Graph](image)

**Fig. 7. Inhibition of trypsin-activated phosphorylase b kinase by EGTA and trifluoperazine at pH 6.8.** Phosphorylase kinase (80 μg/ml) was incubated with trypsin (6 μg/ml) for 30 min before it was diluted to 0.1 μg/ml. Trypsin treatment increased activity 7-fold at pH 6.8. Phosphorylase kinase activity was determined as described under "Experimental Procedures" with 0.2 mM [γ-32P]ATP and no added CDR. The reaction mixtures also included calcium (■), or calcium and 196 mM trifluoperazine (△), or EGTA (□) or EGTA and 190 μM trifluoperazine (△). The ordinate axis displays 32P incorporated per 300 pmol of phosphorylase b.

**Table III**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Method of activation</th>
<th>pH</th>
<th>Specific activity</th>
<th>Stimulation by glycogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.6 mg glycogen/ml</td>
<td>pmol/min/μg/μM</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>6.8</td>
<td>22</td>
<td>145</td>
</tr>
<tr>
<td>2</td>
<td>Protein kinase</td>
<td>6.8</td>
<td>72</td>
<td>323</td>
</tr>
<tr>
<td>3</td>
<td>Trypsin</td>
<td>6.8</td>
<td>91</td>
<td>110</td>
</tr>
</tbody>
</table>

Phosphorylase kinase activity was determined with calcium but in the absence of CDR with or without 1.6 μg of glycogen/ml as described under "Experimental Procedures." In Experiment 2, phosphorylase kinase was phosphorylated to the extent of 2.6 mol/335,000 g of phosphorylase kinase in the presence of the cyclic AMP-dependent protein kinase as described under "Experimental Procedures." In Experiment 3, phosphorylase kinase was activated by incubation for 30 min at 30°C with trypsin. The reaction mixture for activation with trypsin contained 200 μg of phosphorylase kinase/ml, 0.4 μg of trypsin/ml, 50 mM glycerophosphate, 0.9 mM EDTA, 14 mM β-mercaptoethanol, and 3% glycerol.

Terminal tetradecapeptide of phosphorylase b (38). These observations suggest that stimulation of phosphorylase b phosphorylation is substrate-mediated (38). However, glycogen clearly binds to phosphorylase kinase, since it stimulates autophosphorylation as well (9). Furthermore, it does not stimulate phosphorylase kinase which has been treated with trypsin (Table III), suggesting that stimulation by glycogen is mediated at least in part by binding to phosphorylase kinase and that proteolysis destroys these binding sites. Thus, it is possible that stimulation by glycogen involves conformational changes induced by binding glycogen to phosphorylase kinase resulting in greatly increased interaction of the enzyme with phosphorylase b, but not with the tetradecapeptide or with glycogen synthase.

When both glycogen and CDR were included in the phosphorylase kinase assay, trypsin-treated phosphorylase kinase was less active than nonactivated kinase since the stimulation by both glycogen and CDR was nearly abolished with trypsin treatment (Table III). Thus the term "trypsin activation" only applies when phosphorylase kinase is assayed under conditions which exclude glycogen and CDR.

**DISCUSSION**

CDR remains bound to phosphorylase kinase during gel filtration and ion exchange chromatography in the presence of calcium chelators, since purified phosphorylase kinase contains approximately stoichiometric amounts of CDR relative to its other subunits (19). In contrast, cyclic nucleotide phosphodiesterase (12, 13), adenylate cyclase (15), and myosin light chain kinase (14) dissociate from CDR when subjected to similar procedures. The effect of CDR on cyclic nucleotide phosphodiesterase and adenylate cyclase is abolished by phenethylamine derivatives such as trifluoperazine which bind to CDR in the presence of calcium (22). We found that trifluoperazine inhibited nonactivated and phosphorylated phosphorylase kinase at pH 6.8 and at pH 8.2. The fact that native phosphorylase kinase is inhibited by low concentrations of trifluoperazine and is inactive in the absence of calcium is consistent with the hypothesis that the activity of native phosphorylase kinase is regulated by the CDR subunit.

Despite the fact that purified phosphorylase kinase contains CDR, the addition of CDR stimulates the activity of phos-
phorylase kinase 2- to 6-fold at pH 6.8 when the protein substrate is phosphorylase b (Fig. 1), glycogen synthase (Fig. 3), or phosphorylase kinase itself (Fig. 2). The finding that phosphorylation of phosphorylase b is stimulated by CDR is in agreement with the results presented by Cohen et al. (19-21) and by DePaoli-Roach et al. (36). However, DePaoli-Roach et al. (36) found a larger stimulation at pH 8.2 than at pH 6.8. In addition, we demonstrated that CDR stimulates the rate of autophosphorylation and the rate at which purified phosphorylase kinase catalyzes the phosphorylation of glycogen synthase. The fact that CDR increases enzymatic activity when the substrate is phosphorylase b, glycogen synthase, or phosphorylase kinase itself argues against a mechanism in which the stimulation by CDR results from binding to the protein substrate, i.e. substrate-directed effects. Stimulation of phosphorylase kinase activity by CDR is not accompanied by increased sensitivity to phosphorylase b, ATP, or glycogen; instead, the $V_{max}$ of the enzyme is increased. Stimulation of phosphorylase kinase by glycogen differs from stimulation by CDR in that glycogen lowers the $K_m$ for phosphorylase b (i) and stimulates phosphorylated and nonactivated enzyme at pH 8.2 (Table III).

Krebs et al. (1) showed that autophosphorylation is minimal during the phosphorylase b-kinase reaction, presumably due to the low concentration of kinase and the presence of glycerophosphate (5). DeLange et al. (9) further showed that autoactivation of phosphorylase kinase was increased when the kinase concentration was increased. However, we found that the specific activity of the kinase was constant over a 50-fold change in kinase concentration either in the presence or absence of added CDR. Likewise, the phosphorylase kinase reaction rates were generally constant over the course of the reactions either in the presence or absence of added CDR. The stimulation by CDR therefore does not appear to be related to increased autophosphorylation, since the kinase did not become increasingly activated at long incubation times or at high concentrations. Furthermore, autoactivation decreases the $K_m$ for phosphorylase b (1), whereas CDR does not (Table II).

The evidence presented here indicates that stimulation of phosphorylase kinase by CDR involves binding of additional CDR to phosphorylase kinase as shown by the reaction below:

$$a_0 b y_1 CDR + y CDR = a_0 b y_1 CDR + y$$

(1)

According to Equation 1, phosphorylase kinase contains x subunits of CDR which do not dissociate from the enzyme during purification or when the enzyme is diluted into the reaction mixture. The species depicted on the left hand side of the equilibrium is responsible for the calcium-dependent activity observed in the absence of added CDR. The addition of CDR results in reversible binding of y additional molecules of CDR. Cohen et al. (19, 20) have presented evidence that x = 4 but there is no evidence regarding the value for y. We found that the apparent dissociation constant for the binding of added CDR was 15 nm, which is in agreement with the observation of Cohen et al. (20).

It is not known if the sites which bind CDR reversibly are occupied in vivo. If so, dissociation occurs during purification. Binding to these sites may be similar to the reversible binding of CDR to myosin light chain kinase or to cyclic nucleotide phosphodiesterase. The difference between the binding sites for CDR subunits and those sites which bind CDR reversibly is not clear. Perhaps the CDR complexes to equivalent sites which exhibit negative cooperativity with respect to binding. Thus, the last y molecules would bind less tightly than the first x molecules and full activity would require the addition of excess CDR to replace y molecules of CDR lost during purification. Alternatively, there may be two completely different types of binding sites. In accordance with the latter theory, the high affinity sites may be occupied by 4 CDR molecules/tetramer of purified phosphorylase kinase. The low affinity sites, responsible for activation, may be bound to troponin C in vivo as suggested by Cohen et al. (20). A complete understanding of the mechanism of action of CDR will require additional information on the number of binding sites for CDR and the nature in which CDR is bound to these sites.

It has long been recognized that phosphorylase kinase has the potential to be regulated in vivo by phosphorylation and dephosphorylation mechanisms. In addition, enzyme activity can be increased by factors such as glycogen and calcium, which do not cause covalent modification. Since muscular activity is associated with increased intracellular calcium levels the enzyme would be more active under conditions where glycogen breakdown is desirable, i.e. high glycogen levels (41) and muscular activity (11). The recent finding that CDR also increases phosphorylase kinase activity toward glycogen synthase as well as phosphorylase may indicate the existence of additional regulatory mechanisms which couple glycoenerogenesis to muscle contraction. For example, it is possible that the position of the equilibrium described by Equation 1 is shifted in vivo by effectors of phosphorylase kinase, by agents which bind CDR to alter its effective concentration, or by absolute changes in the level of CDR.

REFERENCES

*After this manuscript was submitted, Shenolikar et al. (42) also presented evidence for two types of CDR binding. Their results are in agreement with ours in that the stimulation by CDR was independent of kinase concentration, that half-maximal stimulation was seen at 10 nm CDR, and that trifluoperazine completely blocked the stimulation by CDR but only partially inhibited kinase activity in the absence of CDR. Like DePaoli-Roach et al. (36), they reported calcium-dependent binding of the kinase to CDR-affinity columns.