Isolation of an Autoinhibitory Region from the Regulatory \( \beta \)-Subunit of Phosphorylase Kinase*

(Received for publication, January 7, 1993, and in revised form, May 7, 1993)

Veronica E. Sanchez‡ and Gerald M. Carlson§

From the Department of Biochemistry, College of Medicine, The University of Tennessee, Memphis, Tennessee 38163

An equimolar mixture of the regulatory \( \alpha \)- and \( \beta \)-subunits of phosphorylase kinase has been shown to inhibit its catalytic \( \gamma \)-subunit (Paudel, H. K., and Carlson, G. M. (1987) *J. Biol. Chem.* 262, 11912–11915). The possible presence of an autoinhibitory sequence within those regulatory subunits has been evaluated by peptide isolation and characterization following chemical and proteolytic cleavage of an isolated equimolar mixture of those subunits; the peptides generated were tested for their ability to inhibit the activity of a complex of the \( \gamma \)-subunit and calmodulin. An isolated inhibitory fragment, hereafter referred to as I-peptide, was sequenced and found to correspond to residues 420–436 of the \( \beta \)-subunit (KRNPUGKRFPSNCGRD). This sequence showed homology with the kinase's natural substrate, phosphorylase b. A synthetic peptide based on this sequence was constructed and used to study the mechanism of inhibition. Kinetic analysis of the inhibition of the \( \gamma \)-subunit-calmodulin complex by the I-peptide revealed a competitive pattern versus the homologous substrate phosphorylase b and an uncompetitive pattern versus MgATP, suggesting an ordered binding of substrates, with the nucleotide binding first. In addition to its ability to inhibit, the I-peptide was also a substrate for the \( \gamma \)-subunit-calmodulin complex, with a relatively good \( K_m \) but poor \( V_{\text{max}} \). The parallel inhibition of free \( \gamma \)-subunit and the \( \gamma \)-subunit-calmodulin complex by progressively increasing concentrations of I-peptide provided further evidence that the peptide inhibits by interacting directly with the catalytic subunit and not with the stimulatory calmodulin molecule. The results of this study are consistent with previous findings from this laboratory showing that the conformation of the \( \beta \)-subunit changes following activation of phosphorylase kinase through a variety of mechanisms.

---

* This work was supported by Research Grant DK 32953 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Current address: Product Development Dept., Division Latin America, Procter & Gamble, Caracas, Venezuela.

‡ To whom correspondence should be addressed: Dept. of Biochemistry, College of Medicine, The University of Tennessee, Memphis, Tennessee 38163. Tel.: 901-594-3802; Fax: 901-594-3800.

---

Phosphorylase b kinase (EC 2.7.1.38) catalyzes the phosphorylation of glycogen phosphorylase to generate the 5'-AMP-dependent form of this enzyme (for a review Ref. 1). The holoenzyme of this kinase is composed of four heterologous subunits in a stoichiometry of \( (\alpha_2\beta) \) (2). The activity of phosphorylase kinase is modulated by changes in intracellular free Ca\(^{2+}\) concentration (3), through phosphorylation by cAMP-dependent protein kinase (4), and by allosteric activation by ADP (5).

The amino acid sequences of the kinase's \( \alpha \)- and \( \beta \)-subunits were deduced after cDNA cloning (6, 7). Although sequence comparison reveals extensive regions of homology between the two subunits, they also have unique domains where the known phosphorylation sites are located (7). The \( \gamma \)-subunit has been demonstrated in several independent studies to be the catalytic subunit (8–10), and its primary structure has a significant degree of homology with the catalytic domains of other protein kinases (11, 12). The \( \delta \)-subunit, identical to calmodulin (13), activates the isolated catalytic subunit (9, 10), and is associated with the holoenzyme in a \( \mathrm{Ca}^{2+} \)-independent manner (2).

The mechanism responsible for maintaining several different protein kinases and protein phosphatases in their inactive state is an interaction between the catalytic site and a specific inhibitory region within the same enzyme (14–17). Several lines of evidence suggest that the \( \alpha \)- and/or \( \beta \)-subunits of phosphorylase kinase inhibit the catalytic subunit of the nonactivated holoenzyme. For example, activation is achieved upon proteolytic cleavage of the \( \alpha \)- and \( \beta \)-subunits or upon phosphorylation of those subunits by cAMP-dependent protein kinase (18, 19). After dissociation of the \( \alpha \)- and \( \beta \)-subunits, the \( \gamma \)-subunit-calmodulin complex has a high \( \mathrm{pH} \) 6.8/pH 8.2 activity ratio (8, 20), similar to that of the activated holoenzyme (activation of the holoenzyme is associated with an increase in the ratio of activities at the two \( \mathrm{pH} \) values). An equimolar mixture of the \( \alpha \)/\( \beta \)-subunits inhibits the \( \gamma \)-subunit-calmodulin complex and the \( \gamma \)-subunit (10). In reactivation experiments after SDS-dissociation and electrophoretic separation of the subunits of phosphorylase kinase, the \( \alpha \)- and \( \beta \)-subunits were each able to inhibit the activity of the \( \gamma \)-subunit (21). One interpretation of all these observations is that within the nonactivated holoenzyme, the catalytic subunit is inhibited by constraining quaternary interactions with the regulatory subunits, and that activation results when these constraining interactions are perturbed. The \( \beta \)-subunit seems to have a key regulatory role in this regard. Phosphorylation of this subunit parallels activation (22–24), and specific conformational changes in the \( \beta \)-subunit occur concomitantly with activation by several mechanisms (25–27). The presence of discrete "autoinhibitory" sequences in the regulatory \( \alpha \)- and \( \beta \)-subunits has been evaluated by studying the inhibitory effects of peptides generated from a mixture of \( \alpha / \beta \)-subunits on the activity of the \( \gamma \)-subunit-calmodulin complex. A preliminary account of this work has been presented (28).

**EXPERIMENTAL PROCEDURES**

**Enzymes, Proteins, and Peptides**

Phosphorylase kinase was purified from fast twitch skeletal muscle of New Zealand White rabbits (19), dialyzed against Hepes (50 mm,
Determining of Protein and Peptide Concentrations
The concentrations of phosphorylase kinase, phosphorylase b, and BSA were determined spectrophotometrically using their respective absorbance indices (19, 30, 31). The concentrations of calmodulin and the isolated γ-subunit were determined using the Bio-Rad protein assay with BSA as standard. The concentrations of S-peptide and I-peptide were determined by spectrophotometric absorbance at 210 nm using BSA as standard according to Farrar and Carlson (32).

Renaturation of the γ-Subunit
γ-Subunit-Calmodulin Complex for Screening of Peptide Inhibition
The isolated γ-subunit in urea (8 M), H2PO4(0.1 M), and EDTA (0.1 M), pH 3.3, was renatured overnight at 4°C in the presence of calmodulin. A standard renaturation mixture of 50-μl volume contained the following: γ-subunit (50 μg/ml), calmodulin (50 μg/ml), urea (0.5 M), P (6 mM), EDTA (60 μM), CaCl2 (0.5 mM), dithiothreitol (1 mM), and Hepes (100 mM, pH 8.0). The urea and P were carried over with the isolated γ-subunit. This is the renaturation mixture used in experiments such as those shown in Figs. 1 and 3.

γ-Subunit-Calmodulin Complex for Inhibition Analyses and Kinetic Characterization of I-Peptide as Substrate
A standard renaturation mixture of 50 μl contained: γ-subunit (20 μg/ml), calmodulin (80 μg/ml), BSA (1.66 mg/ml), urea (0.5 M), P (10 μM), EDTA (100 μM), CaCl2 (1 mM), dithiothreitol (1 mM), and Hepes (100 mM, pH 8). This renaturation was carried out overnight at 4°C. These renaturations were used as the starting material for the equimolar mixture of α/β-subunits, as indicated in the appropriate legends. These conditions were used for the activity determination for the kinetic inhibition analysis (Fig. 5).

Cyanogen Bromide Cleavage of the Equimolar Mixture of α- and β-Subunits and Recursed-phase HPLC Isolation of Peak I
The equimolar mixture of α/β-subunits and calmodulin was separated using reverse-phase HPLC on a Delta Pak C8 column (300 Å, 3.9 × 300 mm, Waters Associates) at a flow rate of 1 ml/min. Peptides were eluted with a linear gradient of acetonitrile: 0-35% from 5 to 25 min and 35-60% from 25 to 100 min. Fractions of 1 ml, monitored at 220 nm, were collected and the different peptides were pooled and lyophilized prior to determination of their inhibitory activity. Peak I was re-purified over the same column using the same conditions, and lyophilized prior to sequence analysis.
RESULTS

Isolation of an Inhibitory Peptide from the β-Subunit of Phosphorylase Kinase—Previous studies have shown that the γ-subunit-calmodulin complex and the isolated catalytic subunit are inhibited by an equimolar mixture of the α/β-subunits of phosphorylase kinase (10). In order to determine which regions within these regulatory subunits were responsible for the inhibition, an isolated equimolar mixture of the α/β-subunits was cleaved with cyanogen bromide as described under ”Experimental Procedures.” In a pilot experiment an approximately 18-fold molar excess (digested crude CNBr-peptide mixture inhibited the activity of the γ-subunit alone and the γ-subunit-calmodulin complex to about 60% of their control activities (data not shown), suggesting that CNBr cleavage at the carboxyl side of the methionine residues in the α- or β-subunits did not completely disrupt the inhibitory region(s). Fractionation of the CNBr digest of the α/β-subunits by reversed-phase HPLC using a biphasic linear gradient of acetonitrile revealed two inhibitory peaks (Fig. 1A). The first peak (designated Peak I) eluted at 41% acetonitrile, whereas the second peak (labeled Peak II) eluted at 47% acetonitrile. Peak I was isolated (Fig. 1B) and lyophilized; sequence analysis of this purified inhibitory peptide yielded the sequence (beginning with cycle two of seven cycles) DGVFRG, which corresponds exclusively to a 108-residue CNBr fragment from residues 373 to 480 in the sequence of the β-subunit (Fig. 2). That the autoinhibitory peptide is from the β-subunit is consistent with the importance of this subunit in the regulation of the activity of the holoenzyme. Currently, Peak II is being isolated in sufficient quantities to be sequenced, and we do not yet know if it is derived from the α- or β-subunits. Because of the potential side reactions that can occur during CNBr cleavage of proteins, such as partial deamination (37), it is even possible that Peak II is derived from Peak I.

Further Cleavage and Isolation of the I-Peptide from the CNBr-generated Inhibitory Peptide—In order to delineate the inhibitory region within the large CNBr peptide, the 108-residue peptide from Peak I (Fig. 1) was treated with S. aureus V8 protease as described under “Experimental Procedures.” Initial studies in which the CNBr-peptide mixture from the α/β-subunits was further digested with V8 protease under conditions that ensured complete cleavage at the carboxyl side of both aspartate and glutamate residues indicated that this proteolysis did not affect the inhibitory capacity of the peptide digest (28). The most potent inhibitory fragment (I-peptide) derived from a V8 proteolytic digestion of isolated Peak I eluted at 30 min as a single peak during reversed-phase HPLC (Fig. 3A). This inhibitory peak was further purified (Fig. 3B) and lyophilized; sequence analysis of the purified I-peptide yielded the sequence KRNPGSQ through seven cycles, which corresponds exclusively to a 17-residue fragment from residues Lys-420 to Asp-436 in the sequence of the β-subunit (Fig. 2, underlined residues).

Effect of the I-Peptide on the Free γ-Subunit—the equimolar mixture of the α/β-subunits was able to inhibit the activity of the γ-subunit-calmodulin complex, as well as that of the free γ-subunit (10), and our initial studies indicated that the peptide digest generated by CNBr cleavage of the isolated α/β-subunits inhibited both the γ-subunit-calmodulin complex and the free γ-subunit to approximately the same extent (28). These observations suggested that the inhibition was due to a direct interaction with the catalytic subunit and was not mediated through calmodulin in the γ-subunit-calmodulin complex. To gain more quantitative evidence in support of this hypothesis, we compared the extent of inhibition of the activity of the free γ-subunit and of the γ-subunit-calmodulin complex by identical concentrations of I-peptide. As shown in Fig. 4, progressively increasing concentrations of I-peptide caused parallel inhibition of the free γ-subunit and γ-subunit-calmodulin complex, indicating that the I-peptide inhibits by binding to the γ-subunit and not to calmodulin.

Kinetic Analyses of the Inhibition of the γ-Subunit-Calmodulin Complex by the I-Peptide—The inhibitory properties of the I-peptide were examined using a synthetic version of the inhibitory peptide (for details see “Experimental Proce-

![Fig. 1. Reversed-phase HPLC fractionation of the CNBr-peptide digest of an equimolar mixture of the α/β-subunits of phosphorylase kinase. A, after CNBr cleavage of the α- and β-subunits of phosphorylase kinase, the peptide mixture was resuspended in 0.1% trifluoroacetic acid (aqueous) and subjected to a biphasic gradient in reversed-phase HPLC as described under “Experimental Procedures.” B, repurification of Peak I using the same conditions as above. Peptides were detected by their absorbance at 220 nm, and their inhibitory activity was assayed as under “Experimental Procedures.” The numbers at the top of the peaks represent the relative percentage of inhibition rendered by a fixed volume of each.](image1)

![Fig. 2. An autoinhibitory peptide from the β-subunit of phosphorylase kinase. The figure shows a schematic representation of the primary structure of the β-subunit of phosphorylase kinase with the CNBr cleavage sites denoted by vertical lines. The complete sequence of the 108-residue inhibitory peptide is given using the single letter code for amino acids and corresponds to the CNBr fragment from residues 373 to 480. The I-peptide, generated after S. aureus proteolytic cleavage of Peak I, is underlined and corresponds to residues 420-436 in the amino acid sequence of the β-subunit.](image2)

![Fig. 3. Isolation of I-peptide. A, a V8 peptide digest of isolated Peak I was fractionated by reversed-phase HPLC as described under “Experimental Procedures.” B, repurification of I-peptide. Peptides were detected at 210 nm, and their inhibitory activity was assayed as described under “Experimental Procedures.” The numbers at the top of the peaks represent the percentage of inhibition by a fixed volume of each.](image3)
Fig. 4. Comparison of inhibition by I-peptide of phosphorylase b conversion by the free γ-subunit and by the γ-subunit-calcium modulin complex. For assays of the free γ-subunit, the renatured γ-subunit mixture was diluted 10-fold with Buffer B, and 1 volume of this diluted renaturation mixture was added to 2 volumes of assay mixture and 1 volume of I-peptide to start the reaction. The final concentrations in the 30-min assay at 30 °C were: γ-subunit (0.5 μM/mL), BSA (0.30 mg/mL), CaCl2 (0.50 mM), EGTA (80 μM), Mg(CH2COO)2 (9 mM), [γ-32P]ATP (30 μM), phosphorylase b (5 μM), EDTA (25 μM), P1 (0.25 mM), urea (20 mM), diithiothreitol (0.1 mM), Hepes (100 mM, pH 7), and I-peptide (from 0.5 to 4 mM). For assays of the γ-subunit-calciummodulin complex the concentrations of the assay components were as above, except for the complex, which was as described under “Experimental Procedures.”

Fig. 5. Initial velocity patterns for inhibition by I-peptide. Activity was assayed as described under “Experimental Procedures.” A, inhibition of the γ-subunit-calciummodulin complex by the I-peptide with phosphorylase b as the variable substrate. The final concentrations in the assay were: phosphorylase b (0.11-10.0 μM), I-peptide (0-1.125 mM) and [γ-32P]ATP (110 μM). B, inhibition of the γ-subunit-calciummodulin complex by the I-peptide with ATP as the variable substrate. The concentration of [γ-32P]ATP (ICN) ranged from 12.5 to 100 μM, with I-peptide concentrations varying from 0 to 4 mM, and phosphorylase b was at a fixed concentration of 5 μM.

Fig. 6. Autoinhibitory regions of protein kinases and phosphatases. The reported autoinhibitory regions of several kinases and calcineurin, along with the sequence of the phosphorylatable region of their respective exogenous substrates, are shown. In each case, the top line, or lines, indicates the autoinhibitory sequence in the enzyme, whereas the bottom line shows the area surrounding the convertible region in its substrate. The boxes show conserved amino acids. Pbk, phosphorylase b kinase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; MLCK, myosin light chain kinase; sk, skeletal muscle; sm, smooth muscle. L-PK, liver pyruvate kinase; EGFR, epidermal growth factor receptor; smMLC and skMLC, smooth and skeletal muscle myosin light chain; CaN, calcineurin. The sequence from the γ-subunit of phosphorylase kinase is from this study, the pseudosubstrate region from the γ-subunit of phosphorylase kinase is from Ref. 17, the CaN sequence is from Ref. 50, and all other sequences are taken from Ref. 14.

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>SEQUENCE</th>
<th>SUBSTRATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pbk (γ-subunit)</td>
<td>RDPYALRPL</td>
<td>Phosphorylase b</td>
</tr>
<tr>
<td>Pbk (β-subunit)</td>
<td>QKRFPSNCGR</td>
<td></td>
</tr>
<tr>
<td>PK-A (Type II)</td>
<td>RFDVS</td>
<td>L-PK</td>
</tr>
<tr>
<td>PK-A (Type I)</td>
<td>photons</td>
<td>EGFR</td>
</tr>
<tr>
<td>PK-C</td>
<td>KQAGLRQK</td>
<td>EGFR</td>
</tr>
<tr>
<td>skMLCK</td>
<td>RRRKMDFATGS</td>
<td>skMLC</td>
</tr>
<tr>
<td>smMLCK</td>
<td>RRRKQKTGHAVR</td>
<td>smMLC</td>
</tr>
<tr>
<td>CaN</td>
<td>PRRDAMPS</td>
<td>Pbk (γ-subunit)</td>
</tr>
<tr>
<td></td>
<td>EFRLSIST</td>
<td></td>
</tr>
</tbody>
</table>
modulin complex and found that it was. The $K_v$ value for the I-peptide of 1.19 mM (Table I) was one-eighth of its $K_r$ value. Inasmuch as previous studies have shown that the tetradecapeptide (S-peptide) corresponding to the convertible region of phosphorylase $b$ is a good substrate for the $\gamma$-subunit-caldmodulin complex (32), we compared the kinetic parameters of the S- and I-peptides with those of phosphorylase $b$. Although the $K_v$ value for the I-peptide was 10-fold greater than that for phosphorylase $b$, it was only one-fourth of that for the S-peptide (Table I). The $V_{max}$ value for phosphorylation of the I-peptide was, however, considerably lower than that for either phosphorylase $b$ or the S-peptide (1/10 and 1/20, respectively). As another index of its being a poor substrate, the $V_{max}/K_v$ ratio for the I-peptide was only 1/6 of that for the S-peptide and 1/100 of that for phosphorylase $b$.

**DISCUSSION**

Early studies showed that the remarkably large activation of the phosphorylase kinase holoenzyme by trypsin was associated with proteolysis of only its $\alpha$- and $\beta$-subunits (18, 19). Also, activation of the holoenzyme during phosphorylation by various protein kinases has been correlated with the extent of phosphorylation of the enzyme's $\beta$-subunits (18, 19, 23, 24, 38). In a series of structural studies employing different conformational probes, this laboratory has shown that concomitant with activation of the kinase holoenzyme through various mechanisms (phosphorylation, allosteric activation by nucleoside diphosphates, and preincubation with $Ca^{2+}$ and $Mg^{2+}$ ions), there is a common conformational change in its $\beta$-subunits (5, 25-27). Moreover, this conformational change in the $\beta$-subunits that is associated with activation was still observed following the specific hydrolysis of the holoenzyme's $\alpha$-subunits (26). Based on all of these observations, it was concluded that activation of the kinase holoenzyme is simply deinhibition caused by a conformational change in at least the $\beta$-subunits that releases constraint on the activity of the catalytic $\gamma$-subunits imposed by the quaternary structure of the nonactivated holoenzyme (25, 26, 39). Direct evidence consistent with the idea that the conformation of the $\beta$-subunit regulates the activity of the catalytic $\gamma$-subunit was obtained when it was shown that increasing amounts of an equimolar mixture of $\alpha/\beta$-subunits progressively decreased the activity of reanimated $\gamma$-subunit and that a phosphorylated $\alpha/\beta$-subunit mixture was significantly less inhibitory (10). Our current results raise the possibility that a discrete autoinhibitory locus from residues 420 to 436 of the $\beta$-subunit may inhibit catalytic activity by directly interacting with the active site. The requisite direct interaction between the $\beta$- and $\gamma$-subunits necessary for this regulatory mechanism to be applicable to the nonactivated phosphorylase kinase oligomer has been reported from chemical cross-linking studies on the holoenzyme (40). Of course, proof that this specific possible autoinhibitory mechanism is actually valid must await direct evidence (as from crystallography or cross-linking) that the appropriate regions of these two subunits are, in fact, in direct contact within the nonactivated native enzyme. This same caveat naturally applies to all enzymes for which a specific autoinhibitory region has been proposed without direct evidence that the proposed interactions actually occur within the context of the holoenzyme.

Although our experimental approach that resulted in identification of the inhibitory peptide from the $\beta$-subunit did not rely on any assumptions about the physicochemical nature of a peptide that might give rise to inhibition, potential autoregulatory domains of an enzyme are usually identified based on a search for specific sequences within the enzyme's primary structure that would most likely produce a particular sought-after property. For instance, in the case of a protein kinase, the substrate specificity of the kinase has been used to locate within its primary structure potential pseudosubstrate regions that are inhibitory when screened as synthetic peptides. Alternatively, for protein kinases that are activated by calmodulin, peptides corresponding to kinase sequences predicted to form the basic amphipathic helices associated with calmodulin binding have sometimes been found to inhibit calmodulin-independent or basal activity. This inhibition by a calmodulin-binding peptide is generally interpreted as being indicative of some type of competition in the native enzyme between the active site and calmodulin for the autoinhibitory sequence, although when the kinase also prefers basic substrates, it is difficult to determine in the absence of more direct evidence the extent to which coincidence contributes to the inhibition by the calmodulin-binding peptide simply because it is basic. In some instances the proposed autoregulatory pseudosubstrate and calmodulin-binding regions are adjacent or overlapping (for reviews of autoinhibitory sequences see Refs. 14, 16, and 17). With phosphorylase kinase, a calmodulin-containing protein kinase that is basophilic, both of the above general approaches based on primary structure characteristics have been used previously to identify potential autoinhibitory sequences that are pseudosubstrates or bind calmodulin or both. Calmodulin-binding sequences that effectively inhibit the activated, but not nonactivated, holoenzyme have been reported to occur within the $\beta$- and $\gamma$-subunits, for the former subunit at locations distinct from the inhibitory peptide identified in this study (41). A potential autoinhibitory region corresponding to residues 332-353 of the $\gamma$-subunit (Fig. 6) has been assigned based on its pseudosubstrate sequence (17). This sequence overlaps with one of two high affinity calmodulin-binding regions that have been identified in the $\gamma$-subunit (42). It has been reported that a truncated $\gamma$-subunit with its C-terminal calmodulin-binding domain removed has a 6-fold higher specific activity than the $\gamma$-subunit-caldmodulin complex (43), and it is certainly possible that the $\gamma$-subunit may directly contribute to its own inhibition within the holoenzyme; however, it can be argued that the $\beta$- and perhaps $\alpha$- subunits contribute even more to the autoinhibition of the nonactivated holoenzyme because its dramatic activation by trypsinolysis occurs with degradation of the $\alpha$- and $\beta$-subunits, but without degradation of the $\gamma$-subunit (18, 19). Although the I-peptide from the $\beta$-subunit characterized in this study is not a potent inhibitor, this is not unusual, inasmuch as the peptides corresponding to the pseudosubstrate regulatory domains of cAMP- and cGMP-dependent protein kinases are

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_v$ (mM)</th>
<th>$V_{max}$ (nmol $^{32}P$/mg $\gamma$/min)</th>
<th>$V_{max}/K_v$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-peptide</td>
<td>119 ± 1</td>
<td>15.77 ± 0.02</td>
<td>0.13</td>
</tr>
<tr>
<td>S-peptide</td>
<td>427 ± 47</td>
<td>320 ± 15</td>
<td>0.75</td>
</tr>
<tr>
<td>Phosphorylase</td>
<td>11.9 ± 0.1</td>
<td>158 ± 15</td>
<td>13.28</td>
</tr>
</tbody>
</table>
also not strong inhibitors (reviewed in Ref. 17). The interaction of a free peptide with an active site does not necessarily accurately represent the interaction of the two corresponding regions within the native holoenzyme. Moreover, it also should be noted that the I-peptide was identified by digestion of intact α/β-subunits and the locus of highest affinity has yet to be identified through screening of this region of the β-subunit with overlapping synthetic peptides.

As summarized in Table I, the I-peptide is a substrate for the catalytic subunit of phosphorylase kinase, albeit a poor one with a Vmax value only 1/10 of that for phosphorylase b and a Ks value 10-fold greater, resulting in a specificity constant (Vmax/Ks) of only 1% of that for phosphorylase. Despite its relatively low turnover number, the fact that the Ks value for the I-peptide is less than its Ks value (1/8 as much) indicates that the phosphorylated I-peptide (product) still dissociates from the Michaelis complex more rapidly than does its nonphosphorylated counterpart (substrate) (44). Although we do not know which of the 2 seryl residues in the I-peptide become(s) phosphorylated, we expect the C-terminal one to be the more likely target based on specificity studies with synthetic peptides homologous to the convertible region of phosphorylase b that show a basic residue 3 residues toward the N terminus from the target Ser to be an important determinant (reviewed in Ref. 45). It is this alignment that is much) indicates that the phosphorylated I-peptide (product) accurately represent the interaction of the two corresponding regions of intact α/β-subunits and the locus of highest affinity has yet to be identified through screening of this region of the β-subunit with overlapping synthetic peptides.

In summary, by screening fractionated peptides from a protein digest we have identified a peptide from the β-subunit of phosphorylase kinase that is capable of inhibiting catalytic activity through directly interacting with the active site. This finding is consistent with the possibility that this region of the β-subunit may be a regulatory autoinhibitory locus that directly contributes to the quaternary constraint responsible for the low specific activity of the nonactivated holoenzyme. It is also consistent with the large body of published data discussed above that demonstrates the importance of the conformation of the β-subunit in defining the activity state of this kinase. Nevertheless, direct evidence must be obtained before we can conclude that this region of the β-subunit is truly an autoinhibitory regulator of activity in the phosphorylase kinase holoenzyme. Because of the complexity of this enzyme with one catalytic and three different regulatory subunits, the probability is also great that intersubunit communication among the different regulatory subunits may further influence catalytic activity indirectly.

Acknowledgments—We thank Dr. Dennis Kieck of the University of Tennessee, Memphis and Dr. Hemant Paudel of the University of Calgary, Canada for helpful discussions concerning this work. We are also grateful to Dr. Bill Lane and colleagues of the Harvard Microchemistry Facility for excellent technical assistance in peptide sequencing and amino acid analysis.

REFERENCES
Autoinhibitory Sequence from Phosphorylase Kinase