Domains of Phosphatase Inhibitor-2 Involved in the Control of the ATP-Mg-dependent Protein Phosphatase*

(Received for publication, June 7, 1994, and in revised form, August 31, 1994)

In-Kyung Park and Anna A. DePaoli-Roach:
From the Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202-5122

Inhibitor-2 (I-2) inhibits the free catalytic subunit of type 1 phosphatase (CS1) and controls the cyclic activation/inactivation of CS1 in the ATP-Mg-dependent protein phosphatase complex. We report here the effect of mutations on these two properties of I-2. Substitution of Thr-72 with Ala, Asp, or Glu generated complexes with CS1 that could not be activated. Mutation of Ser-86 did not affect activation by glycogen synthase kinase-3 (GSK-3) alone but impaired synergistic activation by casein kinase II and GSK-3. Mutations in the region between Thr-72 and Ser-86 did not alter the inhibitory potency of I-2 but prevented complete inactivation of CS1. A mutant without the 35 NH2-terminal residues exhibited an IC50 for CS1 200-fold higher than that of wild-type I-2. However, it formed an inactive phosphatase complex with CS1, which was activated by GSK-3. A mutant with the 59 COOH-terminal residues deleted retained full inhibitory activity and formed an inactive complex that could not be activated by GSK-3. We conclude that the NH2-terminal region of I-2 is involved in inhibition, that the sequence between Thr-72 and Ser-86 is necessary for the conversion of CS1 from an active to an inactive conformation, and that the COOH terminus is required for activation by GSK-3. Thus, different functional domains of I-2 may interact with distinct regions of CS1.

Reversible protein phosphorylation is a ubiquitous mechanism for the control of diverse cellular functions (Edelmann et al., 1987; Hunter, 1987; Hubbard and Cohen, 1993), and protein phosphatases have emerged as fundamental components of these regulatory processes (Shenolikar and Nairn, 1991; Bollen and Stalmans, 1992; Munby and Walter, 1983; DePaoli-Roach et al., 1994). Four main classes, PP1, PP2A, PP2B, and PP2C, comprise the majority of cellular serine/threonine phosphatase activity. The type 1 enzymes (PP1) contain a catalytic subunit that is inhibited by the heat-stable protein inhibitors, inhibitor-1 and inhibitor-2 (I-2) (Huang and Glimann, 1976), whereas the type 2 enzymes (PP2) are insensitive to these inhibitors (Ingebritsen and Cohen, 1983). Inhibitor-1 is active as an inhibitor only after phosphorylation by the cyclic AMP-dependent protein kinase whereas I-2 does not require phosphorylation for activity.

*This work has been supported by National Institutes of Health Grant DK36569. 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.

†To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Indiana University School of Medicine, 635 Barnhill Dr., Indianapolis, IN 46222-5122.

The primary structures of inhibitor-1 (Aitken et al., 1982) and I-2 (Holmes et al., 1986a; Park et al., 1994) have been determined, and the inhibitory domain of inhibitor-1 has been localized to residues 9–54 (Aitken and Cohen, 1982). However, attempts to isolate I-2 inhibitory peptides by proteolysis have been less successful, and only one peptide, containing residues 25–114, was shown to retain 2% of the inhibitory activity. Other peptides, including one consisting of residues 1–49, were not inhibitory (Holmes et al., 1986a). Recent work from this laboratory has indicated that the NH2-terminal 65 residues of I-2 are involved in CS1 inhibition (Park et al., 1994).

Four type 1 phosphatase family enzymes have been identified and characterized: the ATP-Mg-dependent (Yang et al., 1980, 1981a), the glycogen/sarcoplasmic reticulum-associated (Stralfords et al., 1985; Hubbard and Cohen, 1989; Tang et al., 1991), the myofibril-associated (Alessi et al., 1992), and a nuclear phosphatase (Beullens et al., 1992). All these enzymes contain a similar catalytic subunit, CS1, and differ in the associated regulatory subunit that targets the phosphatase to different subcellular locations, confers substrate specificity, and regulates activity (Hubbard and Cohen, 1993). I-2 is the regulatory component of the ATP-Mg-dependent phosphatase, which is a cytosolic enzyme. The phosphatase complex has been purified from various sources in an inactive form (Yang et al., 1980; Ballou et al., 1983; Tung and Cohen, 1984; Li et al., 1985) and can also be reconstituted in vitro at equimolar concentrations of CS1 and I-2 (Hemmings et al., 1982; Resink et al., 1983; DePaoli-Roach, 1984). In freshly prepared rabbit skeletal muscle extract, ~30% of the soluble phosphatase phosphatase can be attributed to the ATP-Mg-dependent form (DePaoli-Roach, 1989).

Activation of the ATP-Mg-dependent phosphatase requires phosphorylation of the I-2 by glycogen synthase kinase-3 (GSK-3) (Hemmings et al., 1982; Ballou et al., 1983) also termed protein factor Fx. The phosphorylation site has been identified as Thr-72 (Aitken et al., 1984), and phosphothreonine in I-2 has been detected in vivo (DePaoli-Roach and Lee, 1985; Lawrence et al., 1988). It has been proposed (Ballou et al., 1983; Villa-Moruzzi et al., 1984; Jurgensen et al., 1984) that phosphorylation of I-2 triggers conformational changes to convert the catalytic subunit from an inactive to an active state, which results in activation of the complex. I-2 also undergoes conformational changes following GSK-3 phosphorylation (Picking et al., 1991). Persistence of phosphate at Thr-72 is not required for activity. Indeed, the phosphorylated complex rapidly dephosphorylates itself, and only then does the enzyme become active toward exogenous substrates (Jurgensen et al., 1984; Li et al., 1985; Vandenheede et al., 1985). In the dephosphorylated complex, the catalytic subunit slowly reverts to the inactive state with a rate similar to that observed for the relaxation of I-2.

I-2 exerts two operationally distinguishable effects on CS1, inhibition and inactivation (for a review see Bollen and Stal-
mans (1992)). Inhibition occurs rapidly, is favored by high concentrations of inhibitor (e.g. 10-fold excess over CS1), and is not reversed by phosphorylation but is released by proteolytic destruction of the I-2. Under inhibitory conditions, the CS1 remains in the active conformation. Inactivation, which is essentially the formation of the inactive holoenzyme, can occur at extremely low concentrations of I-2, equimolar with CS1, is slower, and is reversed by phosphorylation of the regulatory I-2 but not by removal of the inhibitor. Inactivation involves conversion of CS1 to an inactive state. This form can also be activated by treatment with trypsin and Mn<sup>2+</sup> (Ballov et al., 1983) in a process that involves degradation of I-2 with subsequent binding of Mn<sup>2+</sup> to CS1 and conversion of the inactive conformation to the active state. Thus, proteolysis in the absence or presence of Mn<sup>2+</sup> allows determination of the conformational state of CS1. Based on these two properties of I-2, two separate binding sites on CS1 have been proposed. One would be a lower affinity site responsible for inhibition and which may overlap with the catalytic site; the second site, of higher affinity, would be responsible for inactivation (Vandenheede et al., 1985; Jurgensen et al., 1984; Kuznetsov and Stalmans, 1984). I-2 is also phosphorylated by casein kinase II (CKII) (DePaoli-Roach, 1984). The modified sites have been identified as Ser-86, -120, and -121 (Holmes et al., 1987). Phosphorylation of wild-type and mutant I-2 polypeptides was carried out in a 25-ml reaction containing I-2, 50 mM Tris-HCl, pH 7.5, 0.1 mM Mn<sup>2+</sup>, 0.2 mM ATP, 2.0 mM Mg<sup>2+</sup>, 2.0 mM Mn<sup>2+</sup> acetate, 0.02% Brij 35, 100 mM NaCl in the presence of PKI (0.5 units/ml), GS3 (0.5 units/ml), or both. After 90 min at 30 °C, the reaction was stopped by addition of 10% trichloroacetic acid, followed by measurement of phosphate activity of appropriately diluted samples. One unit of PKI is the amount of enzyme that incorporates 1 nmol of phosphate/min into a peptide (RRRDDSDDD) at 50 °C.

Assay of Inhibition and Inactivation of CS1 by I-2.—The I-2 inhibitory activity was measured by its ability to inhibit purified CS1 as previously described (DePaoli-Roach, 1984). Reactions contained the assay in a 100-ml reaction containing 5-20 mg/ml CS1, 200 units/ml PKI, 100 mM NaCl, and 0.02% Brij 35 in 25 ml of 25 mM Tris-HCl, pH 7.0, 0.2 mM Mn<sup>2+</sup> acetate, 0.2 mg/ml of bovine serum albumin, and a 100-fold excess of CS1, and the reaction was continued for 5 min at 30 °C. The reaction mixture was then anaerobically centrifuged for 5 min at 20,000 g. To analyze synergistic activation, the reaction was terminated by addition of 1 ml of 10 mM Tris-HCl, pH 7.5, 0.02% Brij 35, 0.5 mM Mg<sup>2+</sup> acetate, 0.5 mM Mn<sup>2+</sup> acetate, 100 mM NaCl, and 0.2 mg/ml of bovine serum albumin, followed by measurement of phosphate activity of appropriately diluted samples. One unit of PKI is the amount of enzyme that releases 1 nmol of phosphate/min from phosphorylase a. One unit of I-2 is defined as the amount of protein required to inhibit by 50% 4 milliunits of CS1.

Assay of Wild-type and Mutant ATP-Mg-dependent Phosphatases.—Assay of ATP-Mg-dependent phosphatase activity of the I-2-CS1 complexes. 5 µl of appropriately diluted samples were incubated in a 20-µl reaction containing 50 mM Tris-HCl, pH 7.0, 0.10 mM EDTA, 0.25 mM bovine serum albumin/ml, 0.05% Brij 35, 0.2% β-mercaptoethanol, 0.2 mM ATP, and 2.5 mM magnesium acetate in the presence or absence of GS3 (14–28 milliunits/ml) for 10 min at 30 °C. 0.03 µl of [γ<sup>32</sup>P]phosphorylase a (20 µCi/µl) was added to a final concentration of 2 mg/ml and 5 mM caffeine. After 10 min, the reaction was terminated by addition of 1 ml of 10 mM Tris-HCl, pH 7.0, 0.02% Brij 35, 0.5 mM Mg<sup>2+</sup> acetate, 0.5 mM Mn<sup>2+</sup> acetate, 100 mM NaCl, and 0.2 mg/ml of bovine serum albumin, followed by measurement of phosphate activity of appropriately diluted samples. One unit of I-2 is the amount of enzyme that releases 1 nmol of phosphate/min from phosphorylase a. One unit of I-2 is defined as the amount of protein required to inhibit by 50% 4 milliunits of CS1.

Experimental Procedures

Construction and Expression of I-2 Mutant Vectors.—The I-2-CS1 pTRCpUCII plasmid, containing the I-2 and CS1 cDNAs in pTRCpUCII (Park et al., 1994) was first linearized at theSacI site, and then centrifuged for 5 min in a microcentrifuge. 30 µl of supernatant were then transferred to a tube containing 40 µl of 10× excess of CS1, and the reaction was continued for 5 min at 30 °C. The reactions were stopped by addition of a 6-fold excess of trichloroacetic acid, followed by measurement of phosphate activity of appropriately diluted samples. One unit of PKI is the amount of enzyme that releases 1 nmol of phosphate/min from phosphorylase a. One unit of I-2 is defined as the amount of protein required to inhibit by 50% 4 milliunits of CS1.
Results

Expression and Characterization of I-2 Mutant Proteins—Previously we have described I-2 mutants, in which the GSK-3 (Thr-72) and the CKII (Ser-86 and Ser-120/121) phosphorylation sites were mutated to Ala, Asp, or Glu and have demonstrated that Ser-86 is the CKII site that potentiates GSK-3 phosphorylation at Thr-72 (Park et al., 1994). A minimal GSK-3 recognition motif was proposed as -SxxXS(P)-, in which the first phosphate is incorporated by another kinase (Fiol et al., 1987, 1988). However, the spacing between Thr-72 and the synergizing Ser-86 site in I-2 is 13 instead of 3 residues. To determine whether there are structural features in the sequence between Thr-72 and Ser-86 (72-TPYHSMIGDDDDAYS-86), required for synergistic phosphorylation, two mutant I-2s were generated. In one mutant, I-2Pro77-81, residues 77-81 (MIGDD) were replaced by five consecutive prolines and in the other, I-2Ala46-204, residues 76-85 (SMIGDDDD) were deleted, generating the minimal GSK-3 recognition motif, -TXXXS(P)-. Both mutants were expressed in E. coli BL21 (DE3) cells and purified by blue-Sepharose CL-GB chromatography from heat-treated E. coli extracts. From 2 liters of BL21 (DE3) cell culture, ~25–50 mg of I-2 with a specific activity of 1.6–1.9 × 10^5 units/mg were purified 3.1.4-fold with ~70% yield. I-2Pro77-81 and I-2Ala46-204 migrated on SDS-PAGE with an apparent M_r of 32,000 (slightly higher than that of the wild type) and 28,000, respectively (Fig. 1). Both mutant proteins exhibited phosphatase inhibitory potency similar to that of wild-type I-2 (Fig. 2A), indicating that the mutations between Thr-72 and Ser-86 had not affected the structural requirements for phosphatase inhibition.

Preliminary studies had suggested that the NH₂-terminal 65 residues of I-2 were involved in inhibition of CS1 (Park et al., 1994). To define this region further, an I-2 mutant was generated in which the first 35 residues were deleted. The I-2Δ21-35 could not be purified by our usual method since it did not bind to the blue-Sepharose column (data not shown). Analysis of the heat-treated extracts by SDS-PAGE indicated that the I-2 protein constituted the majority of the heat-stable proteins (Fig. 1). Therefore, heat-treated extracts containing the I-2 protein were used for subsequent analyses of this truncation mutant. For comparison, wild-type I-2 in heat-treated extracts was used (Fig. 2B). The I-2Δ21-35 mutant behaved on SDS-PAGE as a species of ~26,000 Da (Fig. 1), larger than the deduced M_r of 19,300. NH₂-terminal sequencing showed that the mutant I-2Δ21-35 started at Met-35 followed by Asp-36. The bulky side chain of the Asp residue appeared to prevent excision of the initiation Met (Hirel et al., 1989). The amount of I-2Δ21-35 in the heat-treated cell extracts was determined by densitometric scanning of the Coomassie Blue-stained gel using as standard known amounts of purified I-2 separated on the same gel. Measurement of phosphatase inhibition indicated that I-2Δ21-35 had very little activity. The IC₅₀ for CS1 was ~400 nM, 2 orders of magnitude higher than that of wild-type I-2 (Fig. 2B). These results confirmed that the NH₂-terminal region is required for inhibition of the active CS1.

To assess the role of the COOH terminus of the I-2 protein a deletion mutant, I-2Δ146-204, lacking the COOH-terminal 59 residues was also prepared. I-2Δ146-204 migrated on SDS-PAGE as a 21,600 Da species (Fig. 1), and on a molar basis it exhibited similar inhibitory activity as wild-type I-2, with an IC₅₀ of ~2.0 nM (Fig. 2C).

Analysis of Phosphorylation of I-2 Mutants—To determine the effect of the changes in the I-2 proteins, the mutated polypeptides were subjected to phosphorylation by GSK-3 and CKII individually and in combination. All four mutants, I-2Ala46-204, I-2Δ21-35, I-2Δ146-204, and I-2Pro77-81, were substrates for GSK-3 (Fig. 3). Under the conditions used, phosphorylation of I-2Pro77-81 was three times lower (0.04 mol/m) than that of the wild type I-2 (0.14 mol/m), and phosphorylation of I-2Pro77-81 was 50% higher (0.2 mol/m). The latter result suggests that GSK-3 (a) does not require residues between Thr-72 and Ser-86 and (b) is not dependent on prior phosphorylation even when the minimal GSK-3 recognition motif (-TXXXS(P)-) has been created by mutagenesis. The lower level of phosphate observed in the proline substitution mutant may indicate an altered local structure, which reduces GSK-3 recognition. CKII phosphorylated I-2Pro77-81 and I-2Δ21-35 to a stoichiometry similar to that of the wild type but phosphorylated I-2Ala46-204 and the COOH-terminally truncated I-2 to a lesser extent. Since upstream acidic residues may influence CKII recognition (Kuenzel et al., 1987), it is possible that removal of the four aspartic residues, 80–84, reduces phosphorylation of I-2Ala46-204. When subjected to the combined action of GSK-3 and CKII, the three deletion mutants underwent synergistic phosphorylation (~0.5 mol of phosphate/mol of I-2 over additivity) whereas the I-2Pro77-81 did not. Concomitant with the synergistic phosphorylation, a reduced electrophoretic mobility on SDS-PAGE was observed (Fig. 3, upper panel). These
results suggest that proline residues disrupt the structure necessary for the enhanced recognition by GSK-3 without affecting CKII phosphorylation and that the minimal GSK-3 motif, -TXXS(P)-, is sufficient but not necessary for synergistic phosphorylation of I-2.

Characterization of ATP-Mg-dependent Phosphatase Reconstituted with Wild-type and Phosphorylation Site I-2 Mutants—To study correlations between phosphorylation of I-2 and activation of the ATP-Mg-dependent phosphatase, wild-type and mutant forms of phosphatase were generated by in vitro reconstitution of the complex using purified CS1 and recombinant wild-type or phosphorylation site mutant I-2s. Reconstituted phosphatase complexes (I-2-CS1) were purified by Affi-Gel blue chromatography (Reink et al., 1983) and monitored for GSK-3/ATP-Mg-dependent phosphatase phosphatase activity. Elution profiles of the wild-type and mutant phosphatase are shown in Fig. 4. Free I-2 and CS1 bound to the column and were eluted with 0.5 M NaCl, whereas the phosphatase complexes were not retained. The elution profile of the I-2WT-CS1, containing recombinant wild-type I-2, was similar to that of I-2RSM-CS1, containing rabbit skeletal muscle I-2. The inactive phosphatase complex was present in the flow-through fractions and could be activated by GSK-3. Based on

the amount of CS1 used in the reconstitution, 50–70% of the activity was recovered from the column as the GSK-3-dependent form. To confirm the presence of I-2 in the complexes, fractions from each column were heat-treated to denature CS1, and then the I-2 activity was measured. Two peaks of I-2 activity were detected, one in the flow-through fractions in correspondence with the GSK-3-dependent phosphatase activity and the other in the 0.5 M NaCl eluate (Fig. 4, thick bars). The second peak results from the presence of excess I-2 over CS1. Western blot analysis, with I-2 and CS1 antibodies, of the reconstituted wild type phosphatase and quantitation against known amounts of the two proteins indicated that the two components were present in the first peak at close to equimolar amounts.

Similar elution profiles were obtained with phosphatase complexes containing I-2A728, I-2A868, and I-2A120/121, except that the I-2A868 mutant phosphatase showed a slightly higher spontaneous activity. The I-2A728-CS1 complex could not be activated by GSK-3, but phosphatase activity was detected by trypsin-Mn²⁺ treatment, which degrades I-2 and activates CS1. These results confirm that phosphorylation of Thr-72 by GSK-3 is an absolute requirement for activation of the phosphatase under nondestructive conditions. Substitution of Thr-72 to Asp-72 or Glu-72 did not change the phosphatase inhibitory activity of the I-2 polypeptide (Park et al., 1994). The mutant phosphatase complex containing I-2A868 could be activated by trypsin-Mn²⁺ but not by GSK-3, indicating that an acidic residue at position 72 cannot substitute for phosphothreonine in the activation process (data not shown). The phosphatase complexes containing I-2A8586 or I-2Glu8786 exhibited elution profile and GSK-3 activation similar to I-2WT-CS1 (data not shown) in agreement with the observation that these substitutions could not replace the phosphate group of Ser-86 in the potentiation of GSK-3 phosphorylation.

Analysis of ATP-Mg-dependent Phosphatase Complexes Containing I-2 Deletion and Proline Substitution Mutants—To determine whether the mutations in the sequence between Thr-72 and Ser-86 of I-2 affect reconstitution and activation of the ATP-Mg-dependent phosphatase, mutant phosphatase complexes containing I-2Pro77–81 and I-2Ala76–85 were also reconstituted as described above. Both mutant complexes displayed elution profiles similar to that of the wild-type complex except that the mutant complexes had much higher basal phosphatase activity (Fig. 5). Excess I-2 bound to the column and eluted at 0.5 M NaCl, indicating that the partial inactivation was not due to limiting amounts of the inhibitor protein. Furthermore, excess CS1 would have bound to the resin. GSK-3 activated the mutant phosphatase complexes by only 2-fold, but the total activity was comparable to that of the other complexes. The higher spontaneous activity was unpredicted since both mutants had inhibitory activity similar to that of the wild type (Fig. 2A). However, conditions for inhibition and inactivation are different. Inhibition is almost instantaneous and requires a 10-fold molar excess of I-2 over CS1, whereas inactivation is slower (t½ ~ 15 min) and can occur at equivalent concentrations of the two components. Most likely, inactivation involves a "low affinity" site whereas inactivation involves a "high affinity" site. Therefore, the time course of inactivation of CS1 by I-2Pro77–81 and I-2Ala76–85 was compared with that by wild-type I-2. Determination of phosphatase activity during the reconstitution indicated that up to 5 min, wild-type and mutant I-2 decreased CS1 activity in a similar manner (Fig. 6). However, while I-2 wild type almost completely inactivated CS1 in a time-dependent manner, both the I-2Pro77–81 and I-2Ala76–85 inactivated only by ~50%. A 60-min reconstitution experiment with a CS1 to I-2 molar ratio of 1:1.5 yielded essentially the same results (data not shown). Therefore, the high spontaneous
activity observed after the Affi-Gel blue chromatography of the complexes is not due to an artifact but reflects the formation of partially inactive phosphatases. The similar initial rate of reduction of CS1 activity may result from inhibitory interactions that are not affected by these mutations. Support for this hypothesis comes from the finding that, as previously reported (Hemmings et al., 1982; Vandenheede and Merlevede, 1985), GSK-3 cannot reactivate the phosphatase at the earlier times but was effective at later times of reconstitution (data not shown). The observation that I-2Pro77-81 and I-2A76-85 could not completely inactivate CS1 in the reconstitution reaction indicates that the sequence between Thr-72 and Ser-86 is involved in the changes in conformation of CS1 responsible for induction of the inactive state even though it is not required for inhibition.

Comparison of the time course of activation of phosphatase complexes containing the Pro77-81, Δ76-85, and wild type I-2 (Fig. 7) showed that both mutant complexes could be activated at rates comparable to wild type. However, since the mutant complexes were already partially active, they reached maximal activity sooner. In addition, the mutants reverted more rapidly to the inactive state. This was not due to instability of the enzymes since the basal activity was unchanged throughout the incubation period.

Since I-2Δ1-35 displayed very low inhibitory activity, it was of interest to determine whether it could interact with CS1. The Affi-Gel blue column elution profile demonstrated that an inactive ATP-Mg-dependent phosphatase was recovered in the flow-through fraction which was almost completely dependent on GSK-3 binding to the column, whereas the form complexed with CS1 binds to the column, whereas the form complexed with CS1.

Fig. 3. Phosphorylation of wild-type and mutant I-2. Purified wild-type and mutant I-2 proteins were phosphorylated by GSK-3 (0.5 unit/ml), CKII (0.5 unit/ml), or both, in a 25-μl reaction mixture as described under "Experimental Procedures." 20-μl aliquots were separated on 13% SDS-polyacrylamide gels except for I-2Δ146-204, which was separated on a 15% SDS-polyacrylamide gel. The upper panels show Coomassie Blue-stained gels of phosphorylated I-2, and the lower panels are the corresponding autoradiographs. mol/mol indicates the stoichiometry of I-2 phosphorylation. Molecular size markers are shown in kDa.

Fig. 4. Affi-Gel blue chromatography of wild-type and phosphorylation site mutant ATP-Mg-dependent phosphatases. Purified CS1 (5 μg) and I-2 proteins (5 μg) were incubated for 60 min at 30 °C and then applied onto a 1-ml Affi-Gel blue column. Flow-through fractions were collected, and bound proteins were eluted with 0.5 M NaCl (arrows). Phosphatase activity in the fractions was measured after preincubation in the presence (closed circles) or absence (open circles) of GSK-3 (15 milliunits/ml). For I-2Ala12-CS1, fractions were also preincubated in the presence (closed squares) or absence (open squares) of trypsin plus Mn²⁺ before phosphatase assays. Aliquots of the fractions were heated for 5 min at 100 °C before I-2 activity was determined. The position of I-2 elution is indicated by the bars in each panel. Elution of free CS1 and I-2 is shown in the first panel. RSM indicates I-2 purified from rabbit skeletal muscle, and WT indicates recombinant I-2.
complex had been formed that could no longer be activated by GSK-3. These results indicate that although the 35 NH₂-terminal residues are required for inhibition, they are not involved in the formation of the inactive ATP-Mg-dependent phosphatase or in the activation process. The 59 COOH-terminal amino acids, in contrast, although not necessary for inhibition, are important for induction of the active conformation of CS1 following phosphorylation by GSK-3.

**Activation of Wild-type and Mutant ATP-Mg-dependent Phosphatases by Trypsin and/or Mn²⁺ Treatment**—An alternative way of activating the ATP-Mg-dependent phosphatase is by exposure to limited trypsin and Mn²⁺ (Ballou et al., 1983). In this procedure, trypsin degrades the regulatory I-2 component, and then Mn²⁺ activates the inactive CS1. Thus, trypsin treatment alone can indicate whether CS1 is in an active state or not. Mn²⁺ alone has only a modest effect, presumably because the binding site on CS1 is blocked by the I-2. When the purified wild-type and mutant reconstituted ATP-Mg-dependent phosphatases (Table I) were subjected to trypsin alone, no significant effect on the activity of any of the complexes tested was observed, supporting the contention that CS1 was in the inactive state in the holoenzymes. Mn²⁺ alone elicited ~30% of the activity released by the combined action of trypsin and Mn²⁺ (Fig. 5) for the wild type, I-2Pro77-81, and I-2Δ76-85 complexes but fully activated the I-2Δ146-204 and I-2Δ1-35 complexes. A possible explanation is that truncation at either end of the I-2 protein might have exposed a Mn²⁺-binding site in CS1, which is masked by the intact I-2. Since the free inactive CS1 is also fully activated by Mn²⁺ alone, it is possible that both the NH₂ and COOH termini of I-2 somehow affect metal ion binding.

**CKII Potentiation of the Activation of Wild-type and Mutant ATP-Mg-dependent Phosphatases by GSK-3**—To determine whether synergistic phosphorylation of I-2 by CKII and GSK-3 correlated with synergistic activation, the purified wild-type and mutant ATP-Mg-dependent phosphatases were reconstituted with increasing amounts of GSK-3 in the presence or absence of CKII prior to measurement of phosphatase activity (Fig. 7). All phosphatase complexes tested were activated by GSK-3 in a dose-dependent manner. CKII enhanced, up to 2-fold, the GSK-3 activation of the complexes containing rabbit skeletal muscle I-2, recombinant wild-type I-2, and I-2Ala120/121. In these cases, synergistic phosphorylation also occurred (Park et al., 1994). In agreement with previous observations (DePaoli-Roach, 1984), CKII itself has no effect on phosphatase activity, and synergistic activation was observed only at submaximal concentrations of GSK-3. Consistent with the lack of

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**Fig. 5.** Affi-Gel blue chromatography of ATP-Mg-dependent phosphatase reconstituted with I-2 proline substitution and deletion mutants. The mutant ATP-Mg-dependent phosphatases were reconstituted using purified CS1 (5 μg) and I-2Pro77-81 (4.5 μg), I-2Δ76-85 (5 μg), I-2Δ1-35 (5.9 μg), or I-2Δ146-204 (5 μg) and processed as described in the legend of Fig. 4. The phosphatase activity in the fractions was measured after preincubation in the absence (open circles) or presence (closed circles) of GSK-3 (10–20 milliunits/ml) or in the absence (open squares) or presence (closed squares) of trypsin plus Mn²⁺. I-2Δ1-35 was quantitated by Western immunoblotting using anti-I-2 antibody as described under "Experimental Procedures."
potentiation of GSK-3 phosphorylation, no enhanced activation by CKII was observed with complexes reconstituted with I-2Ala72, I-2Ala72, and I-2Pro77–81. However, in spite of the synergistic phosphorylation of the free I-2Δ76–85 mutant, the activation of the complex containing this mutant was not potentiayed by CKII. Therefore, not only phosphorylation of both Thr-72 and Ser-86 but also the intact sequence between these two phosphorylation sites are required for synergistic activation of the ATP-Mg-dependent phosphatase.

**DISCUSSION**

In this study, we examine the correlation between I-2 phosphorylation and activation of the ATP-Mg-dependent phosphatase, and present evidence that phosphorylation is necessary but not sufficient for regulation of the phosphatase complex and that other I-2 structural features are required. We have identified three domains of I-2 that are involved in distinct functions (Fig. 9A). The NH2 terminus participates in inhibition of active CS1, the region between Thr-72 and Ser-86 is involved in synergistic phosphorylation and activation of the phosphatase complex, and the COOH-terminal domain is required for activation by phosphorylation.

GSK-3 substrates fall into two classes (Roach, 1991; Wang et al., 1994), one group in which prior phosphorylation by another kinase is a prerequisite and a second set for which previous phosphorylation is unnecessary. With the GSK-3α iso-
Fig. 9. Model of reconstitution and activation of the ATP-Mg-dependent phosphatase. A, schematic diagram of the functional domains of I-2. The dotted bar indicates the inhibitory domain, the hatched bar the activation domain, and the black bar the region involved in inactivation of CS1 and synergistic phosphorylation and activation of the phosphatase. The 1-2 phosphorylation sites are also shown. B, model for reconstitution and activation of the ATP-Mg-dependent phosphatase. Conformational states of CS1 (C) at each step are indicated at the left side. The different conformations of CS1 are represented by different shapes and shades. WT indicates wild type.

sion to the inactive state but only to inhibition. Our data do not support this latter idea. In fact, trypsin treatment alone did not result in activation, as would be expected if the CS1 was still in the active conformation. Instead, full activity was revealed by incubation of the complex with Mn²⁺ alone. This indicates that the CS1 is in an inactive state but that the metal binding site is no longer masked by the truncated I-2 component. Similar results were obtained with the complex containing the NH₂-terminally truncated I-2, although this phosphatase could be activated by GSK-3. These results may provide an explanation for earlier reports (Vandenheede et al., 1981; Yang et al., 1981b) that inactive ATP-Mg-dependent phosphatase purified from mammalian tissues could be almost fully activated by Mn²⁺ alone. As previously suggested (Cohen, 1989; Bollen and Stalmans, 1992), such preparations could have contained partially proteolyzed I-2.

Deletion of the NH₂-terminal 35 residues of I-2 reduces the potency of inhibition of CS1 by 2 orders of magnitude, with no effect on its ability to inactivate. The complex containing this mutant was activated by GSK-3 similarly to the wild type. Therefore, the NH₂-terminal inhibitory domain of I-2 can be distinguished from the regions involved in inactivation. Holmes et al. (1986a) had reported that a peptide comprising residues 1–49 had no phosphatase inhibitory activity whereas a peptide consisting of residues 25–114 had 2% activity after 10 min of preincubation but 90% inhibition after 60 min. The lack of inhibitory activity of the NH₂-terminal peptide is difficult to reconcile with our results, unless the conformation of the NH₂-terminal region of I-2 in the intact protein is different from that of the short peptide or else other contacts are required for inhibition. One possibility is that the interactions of I-2 involved in inactivation facilitate binding of the inhibitory, NH₂-terminal sequence. The time dependence of inhibition described for the 25–114 fragment most likely reflects what we define as the inactivation process. This idea is also supported by the observation that 20% reactivation was achieved after GSK-3 phosphorylation. The 25–114 peptide does contain the sequence between residues 72 and 86, which our data suggest is involved in inactivation of CS1.

The observation that I-2 has separate regions for inhibition and inactivation of CS1 is consistent with previous proposals for two different I-2 binding sites on CS1 (Jurgensen et al., 1984; Vandenheede and Merlevede, 1985; Vandenheede et al., 1985; Vandenheede et al., 1989). Based on the data presented, we propose a model for interactions of I-2 with CS1 (Fig. 9B). Two types of interactions take place in the formation of the ATP-Mg-dependent phosphatase from separated subunits: a rapid inhibition of CS1 and a slower inactivation. The NH₂-terminus of I-2 is involved in inhibition of CS1 but not in inactivation (Fig. 9). When the first 35 residues were deleted, the mutant could no longer inhibit CS1 but could still inactivate it, forming an inactive phosphatase complex that could be
activated by GSK-3. Therefore, the NH₂-terminal region of I-2 may interact with the low affinity inhibitory site in CS1. The sequence between Thr-72 and Ser-86 is implicated in several functions. First, it is involved in the conversion of CS1 into an inactive conformation. Mutations in this region generated I-2s with normal phosphatase inhibitory activity but lacking the ability to fully inactivate CS1, thus forming partially active complexes. This part of the molecule may interact with the high affinity inactivation site in CS1. The segment 72–86 also appears to be required for synergistic activation of the phosphatase by CKII and GSK-3. When this region was mutated, correlation between synergistic phosphorylation of I-2 and synergistic activation of the phosphatase was lost. The COOH-terminal 59 residues are absolutely necessary for the GSK-3-dependent activation process. Deletion of the COOH terminus does not affect inhibition or inactivation but leads to the formation of an irreversibly inactive phosphatase. Our model predicts multiple contact points between I-2 and CS1. Since inhibition is a very rapid process, it is possible that the initial event in the formation of the I-2-CS1 complex is an interaction between the NH₂-terminal, inhibitory domain of I-2 with the CS1 low affinity inhibitory site. As depicted in Fig. 9B, subsequent interaction with the region around Thr-72 and Ser-86 would result in induction of the CS1 inactive conformation. Upon GSK-3 phosphorylation, a change in the conformation of I-2 would lead to release of the inhibitory domain and conversion of CS1 to the active conformation. The activated phosphatase dephosphorylates itself, thus allowing expression of activity toward exogenous substrates. The complex then reverts slowly to the inactive state by a process which may or may not involve reestablishment of interactions with the inhibitory domain. It is possible that, once the CS1 has acquired its inactive state, I-2 no longer interacts with the CS1 inhibitory site. However, if, in these cases, addition of I-2 to the inactive complex would result in binding of I-2 to the inhibitory site, which should inhibit activation of the complex. Since excess I-2 does not affect the initial rate of activation (Jurgensen et al., 1984), it is unlikely that the inhibitory site is available in the inactive complex. Most likely, upon phosphorylation and activation of the phosphatase, the I-2 inhibitory domain is released, and the complex and involves multiple contacts, some of which we have been able to distinguish in terms of contributions to inhibition, inactivation, and activation of the ATP-Mg-dependent phosphatase. Based on its properties (Foulkes and Cohen, 1980), the heat-stable I-2 polypeptide has been considered to have little structure in solution, but the present study implies that in combination with the catalytic subunit it may acquire a greater degree of structural organization. Exact definition of this structure as well as the details of the I-2-CS1 interactions will have to await the determination of the three-dimensional structure of the complex.

Acknowledgments—We thank Dr. Peter J. Roach for discussions during the course of this work and for criticisms of the manuscript. Peptide sequencing and synthesis of the oligonucleotides were carried out at the Biochemistry Biotechnology Facility at Indiana University.

REFERENCES

ATP-Mg-dependent Protein Phosphatase