Dephosphorylation of Catalytic Subunit of cAMP-dependent Protein Kinase at Thr-197 by a Cellular Protein Phosphatase and by Purified Protein Phosphatase-2A*

Susanne Liauw and Robert A. Steinberg†

From the Department of Biochemistry and Molecular Biology, the University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190

Thr-197 phosphate is essential for optimal activity of the catalytic (C) subunit of cAMP-dependent protein kinase enzyme, and, in the C subunit crystal structure, it is buried in a cationic pocket formed by the side chains of His-87, Arg-165, Lys-189, and Thr-195. Because of its apparent role in stabilizing the active conformation of the C subunit and its resistance to several phosphatases, the phosphate on Thr-197 has been assumed to be metabolically stable. We now show that this phosphate can be removed from C subunit by a protein phosphatase activity extracted from S49 mouse lymphoma cells or by purified protein phosphatase-2A (PP-2A) with concomitant loss of enzymatic activity. By anion-exchange chromatography, inhibitor sensitivity, and relative activity against glycogen phosphorylase a and C subunit as substrates, the cellular phosphatase resembled a multimeric form of PP-2A. PP-1 was ineffective against native C subunit, but it was able to dephosphorylate Thr-197 in urea-treated C subunit. Accessibility of Thr-197 phosphate to the cellular phosphatase was enhanced by storage of C subunit in a phosphate-free buffer or by inclusion of modest concentrations of urea in the reactions and was reduced by salt concentrations in the physiological range and/or by amino-terminal myristoylation. It is concluded that a multimeric form of PP-2A or a related enzyme from cell extracts is capable of removing the Thr-197 phosphate from native C subunit in vitro and could account for significant turnover of this phosphate in intact cells.

Catalytic (C)1 subunit of cAMP-dependent protein kinase when isolated from animal tissues is phosphorylated at two sites, Thr-197 and Ser-338. The recombinant protein expressed in Escherichia coli is phosphorylated at these sites and, in addition, at Ser-10 and Ser-139 (1). Phosphorylation at Thr-197 slows the mobility of C subunit in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and “activates” the protein by reducing its $K_m$ values for both ATP and peptide substrates (2).

Thr-197 falls in the “loop” region between subdomains VII and VIII (3), which also is associated with activating phosphorylation sites in a number of other protein kinases, including CDC2 kinase, MAP kinase (or ERK), MAP kinase kinase (or MEK), and many protein tyrosine kinases (3–5).

In the crystal structure of C subunit, the Thr-197 phosphate appears to stabilize the active configuration of the catalytic cleft through ionic and hydrogen-bonding interactions with the side chains of His-87, Arg-165, Lys-189, and Thr-195 (6). Comparisons of the structures of inactive forms of the CDC2 and MAP kinases with that of the active C subunit suggest that phosphorylation of sites in this “activation loop” region is responsible for a structural rearrangement of the catalytic cleft that promotes substrate binding and catalysis (4, 7). Nevertheless, while phosphorylation of the activation loop in CDC2 kinase, MAP kinase, MAP kinase kinase, and many of the protein tyrosine kinases is regulated by the antagonistic actions of specific protein kinases and protein phosphatases, Thr-197 phosphate in C subunit has been thought to be metabolically stable (8–10).

While undertaking experiments to optimize conditions for immunoprecipitation of C subunit from extracts of S49 mouse lymphoma cells, we noted that incubation of radiolabeled, recombinant C subunit with cell extract resulted in apparent dephosphorylation manifested by an increase in SDS-PAGE mobility. Although this reaction was most dramatic in buffers containing SDS or urea, there was also reactivity in the absence of these agents. This report describes experiments that identify the cellular activity as a protein phosphatase with properties similar to those of a multimeric form of protein phosphatase-2A (PP-2A) and characterize its reaction on C subunit.

**EXPERIMENTAL PROCEDURES**

**Materials**

Chemicals and Biochemicals—Phosphorylase a, glycogen (type III from rabbit liver), o-d-glucose 1-phosphate, ammonium molybdate, 1-amino-2-naphthol-4-sulfonic acid, and Sephadex G-50 were from Sigma; okadaic acid was from L.C. Laboratories; $[\gamma-32P]ATP$ (3000 Ci/mmol) was from DuPont NEN; and ultrapure urea was from ICN Biomedicals. Kemptide, the heptapeptide substrate for C subunit, was prepared and purified by the Molecular Biology Resource Facility of the University of Oklahoma Health Sciences Center. The catalytic subunit and AC complex of PP-2A were gifts from Dr. Marc Mummy (University of Texas Southwestern Medical Center), and recombinant PP-1 and purified inhibitor-2 were gifts from Dr. Ernest Lee (University of Miami School of Medicine). Other chemicals were reagent grade or better and used without further purification.

Solutions—“C subunit storage buffer” contained 100 mM MES (pH 6.5), 10 mM potassium phosphate, 0.1 mM EDTA, 2 mM dithiothreitol, and 50% glycerol. Extraction buffer was 20 mM Tris hydrochloride (pH 7.4), 0.5 mM EDTA, and 10 mM 2-mercaptoethanol. QMA buffer contained 10 mM Tris hydrochloride (pH 7.4), 0.1 mM EDTA, and 0.1% (v/v) 2-mercaptoethanol. Phosphatase buffer was 10 mM Tris hydrochloride...
Dephosphorylation of cAMP-dependent Protein Kinase C Subunit

Methods

Expression and Purification of Recombinant C Subunits—Recombinant murine Cα subunit was expressed from the pET-8c expression vector in E. coli BL21(DE3) and purified as described previously (2). Myristoylated C subunit was prepared in similar fashion from bacteria carrying the C subunit plasmid and pB131, an expression plasmid for yeast N-myristoyl transferase (11). Nonegytaturated C subunits were purified from cells induced for either 50 min or 5 h at room temperature to give either mostly nonphosphorylated C subunit or C subunit fully phosphorylated on Thr-197 (2); myristoylated C subunit was purified from cells induced for 5 h. For most experiments purified C subunits were stored at ∼20 °C in C subunit storage buffer. For assays of anion-exchange column fractions, we used a C subunit preparation that had been stored in buffer containing 100 mM Tris acetate (pH 7.5), 150 mM sodium chloride, 50 mM 2-mercaptoethanol, 0.1 mM EDTA, and 50% glycerol. After several weeks of storage, this preparation had lost about 80% of its activity and became markedly more susceptible to phosphatase-mediated dephosphorylation of Thr-197. Although these changes appeared to be irreversible, there was no evidence of proteolysis, and the new properties were stable to further storage.

Extraction of Protein Phosphatase Activity from S49 Cells—Kinase-negative S49 mouse lymphoma cells (subline 24.6.1 [12]) in mid-logarithmic phase growth were harvested by centrifugation, washed twice by resuspension in phosphate-buffered saline, resuspended at about 4 × 10^9 cells/ml with extraction buffer, frozen on dry ice, and stored at −70 °C. For crude cell extracts, cells were thawed and centrifuged at 4 °C for 15 min at 11,000 × g in a Beckman Microfuge II microcentrifuge. Supernatant fractions were then purified by gel filtration on superfine Sephadex G-50 in extraction buffer. The excluded peaks were concentrated using “Centricon-10” centrifugal concentrators (Amicon), frozen in small aliquots, and stored at −70 °C.

For anion-exchange purification, cell suspensions as above were thawed and centrifuged at 4 °C for 1 h at 100,000 × g. The supernatant fractions were then loaded onto columns of Acelul Plus QMA (500 A, Waters/Millipore) equilibrated with QMA buffer; about 1 ml of packed resin was used for each 7 mg of cell protein. Columns were washed with five volumes of QMA buffer and then eluted with 13.5 column volumes of a linear 0–50 mM sodium chloride in QMA buffer. Fractions containing protein phosphatase activity against C subunit were pooled and concentrated to about 12–15 mg/ml using Centriplus and Centricron concentrators (Amicon), and small aliquots were frozen and stored at −70 °C.

Assay of Glycogen Phosphorylase Phosphatase Activity—Phosphorylase phosphatase was assayed by the colorimetric procedure described by Gruppuso et al. (13) with slight modifications. Equal volumes of diluted phosphatase and phosphorylase a (3 mg/ml) in phosphorylase buffer were mixed and incubated for 60 min at 37 °C. Reactions were stopped by the addition of 49 volumes of ice-cold phosphorylase buffer, and portions of the diluted phosphorylase samples were then assayed for 30 min at 37 °C in the direction of glycogen synthesis after mixing with equal volumes of phosphorylase substrate solution. Released phosphate was measured as the reduced phosphomolybdate after incubation with acidic ammonium molybdate and ammonium molybdate sulfonic acid as described by Fiske and Subbarow (14).

Assay of C Subunit Phosphatase Activity—Equal volumes of diluted phosphatase and purified C subunit in phosphorylase buffer (with or without additions) were incubated at 30 or 37 °C as described in figure legends. (Reactions with PP-1 all included 0.1 mM manganese chloride, without which the enzyme was virtually inactive. Manganese ions had no effect when mixed with either PP-2A or PP-2C.) Reactions were stopped by adding a large excess of SDS gel sample buffer (15) and heating to 100 °C for 2 min. The extent of Thr-197 dephosphorylation was determined by the proportion of C subunit converted to a faster migrating species in SDS-PAGE (2). For the experiments of Figs. 2, 9, and 10, samples were stopped by diluting with phosphatase buffer containing either 100 mM sodium fluoride (see Fig. 2) or 1 mM okadaic acid (see Figs. 9 and 10) and assayed for protein kinase activity using kemptide as substrate as described previously (2).

Gel Electrophoresis, Silver Staining, and Quantitation of Optical Density in Phosphorylated and Nonphosphorylated Forms of C Subunit—SDS-PAGE was carried out as described by Laemmli (16) using 10% polyacrylamide gels with the pH of the lower gel solution optimized for separation of the Thr-197 phosphorylated and nonphosphorylated forms of C subunit as described previously (2). Gels were fixed with trichloroacetic acid, stained with silver nitrate (17), and dried between clear dialysis membrane sheets (Bio-Rad). Dried gels were scanned with a Molecular Dynamics model 300A computing densitometer and quantified using the IQ software package (Molecular Dynamics) in “area scan” mode with manual base line and peak selection. For the scan data of Fig. 2, the raw absorbance data was exported into the FigP software package (BioSoft) and normalized to a common non-C subunit peak; the absorbance in a contaminating species from the C subunit preparation that co-migrated with nonphosphorylated C subunit was subtracted from all scans before integrating.

Radiolabeling and Phosphoamino Acid Analysis—Recombinant, nonphosphorylated C subunit was labeled with [32P]phosphate from [γ-32P]ATP as described previously (2) and dialyzed against two changes of protein-free phosphate buffer using the micro-drop method of Marusyk and Sergeant (18). Portions of the dialyzed sample were incubated without or with protein phosphatase preparations as described in the legend to Fig. 3, mixed with SDS-gel sample buffer, and subjected to SDS-PAGE. Acid hydrolysis of the gel-purified, radiolabeled C subunits and analysis of the hydrolysates by thin layer electrophoresis were as described previously (2).

RESULTS

Extracts from S49 Mouse Lymphoma Cells Is Dephosphorylated C Subunit at Thr-197—Fig. 1 shows the effect of incubation with an S49 cell extract on the SDS-PAGE mobility of purified recombinant C subunit. The purified C subunit gave a single band at about 49 kDa as expected for protein fully phosphorylated at Thr-197 (Fig. 1, lane b). This mobility was unaffected by mixing with the cell extract (Fig. 1, lane c). After incubation for 3 h at 37 °C, the majority of the C subunit was shifted to a form with faster mobility (Fig. 1, lane d). The chelators EDTA and EGTA had no effect on this mobility shift (Fig. 1, lanes e and f), but the protein phosphatase inhibitors sodium fluoride and okadaic acid completely prevented it (Fig. 1, lanes g and h). Sodium phosphate was a weak inhibitor of the “shift activity,” and sodium vanadate was totally ineffective (data not shown). The inhibition by sodium fluoride was not reproduced by sodium chloride (Fig. 1, lane i), indicating that the effect was not simply a response to high ionic strength.

The mobility shifts illustrated in Fig. 1 were consistent with dephosphorylation at Thr-197. The “shifted” form co-migrated with purified “fast-form” C subunit, which is not phosphorylated at Thr-197 (2), and the fast-form preparation was unaffected by incubation with cell extract (data not shown). Since phosphorylation at Thr-197 has a dramatic effect on C subunit activity, we next tested whether or not the putative dephospho-

2 S. Lliauw and R. A. Steinberg, unpublished observations.
Incubation of fast form C subunit with activity indeed removed phosphate from Thr-197 of C subunit.

Extract. We established in early experiments, however, that avoid the complication of C subunit endogenous to the cell used extracts from a kinase-negative mutant of S49 cells to inactivation that was not affected significantly by the inhibitor.

For these and other experiments described in this report, we established in early experiments, however, that wild-type S49 cells contain the same phosphatase activity at about the same level (data not shown).

The experiment of Fig. 3 demonstrated that the S49 cell activity indeed removed phosphate from Thr-197 of C subunit. Incubation of fast form C subunit with [γ-32P]ATP in the presence of trace amounts of "slow form" C subunit results in phosphorylation of the C subunit on Thr-197 and several serine residues (2). Fig. 3A shows gel patterns from such a preparation incubated without or with cell extract or a partially purified phosphatase fraction from S49 cells (below). The radiolabeled C subunit was mostly the slow form species, consistent with efficient phosphorylation at Thr-197.

Treatment with either of the phosphatase preparations reduced the amount of label in C subunit and shifted the labeled protein to the position of fast form C subunit. Fig. 3B shows that the untreated, labeled C subunit had radioactivity in both phosphoserine and phosphothreonine but that the C subunit treated with cell extract or phosphatase had radioactivity only in phosphoserine.

The S49 Cell Protein Phosphatase Active on C Subunit Is Either PP-2A or a PP-2A-like Enzyme—The results described above (Fig. 1) suggested that the S49 cell activity was related to either PP-1 or PP-2A. For further characterization of the activity, we fractionated cell extracts by anion-exchange chromatography. Fig. 4 shows a representative pattern from one of three such separations. Using glycogen phosphorylase a as substrate, we found multiple overlapping peaks of activity that eluted at salt concentrations between about 100 and 300 mM. The phosphatase active on C subunit appeared to co-elute with a portion of the phosphatase active on glycogen phosphorylase. The soluble proteins from an extract of kinase-negative S49 cells were concentrated for use in the experiments of Figs. 3, 5, and 7.

The soluble proteins from an extract of kinase-negative S49 cells were fractionated on a column of AcOid-Plus QMA as described under "Experimental Procedures." Fractions were assayed for absorbance at 280 nm (thick solid line), sodium chloride concentration (by conductivity thin straight line), and protein phosphatase activity on glycogen phosphorylase a (dotted line) or C subunit (dashed line). To enhance sensitivity of the gel-shift assay for C subunit, a preparation of C subunit was used that had increased susceptibility to phosphatase ("Experimental Procedures"). For purposes of scaling, the phosphatase data were normalized to values for peak fractions.

Fig. 3. The S49 cell activity can remove phosphothreonine from autophosphorylated C subunit. Recombinant C subunit was phosphorylated in the presence of [γ-32P]ATP and dialyzed to remove the unreacted ATP as described under "Experimental Procedures." Equal portions of the labeled material were then incubated for 1.5 h at 37°C without (lane a) or with 7 mg/ml of partially purified S49 cell phosphatase (lane b) or 4 mg/ml of a gel-filtered S49 cell extract (lane c). The samples were then subjected to SDS-PAGE, and the labeled C subunits excised from gels for hydrolysis and phosphoamino acid analysis. A shows autoradiographic patterns from analytical SDS-PAGE of the samples before hydrolysis, and B shows thin layer electrophoresis patterns of the hydrolyzed samples. Positions of the two forms of C subunit are indicated in A as for Fig. 1; positions of internal phosphoserine (Ser-P) and phosphothreonine (Thr-P) standards are shown for B.
to each other and of about the same order of magnitude as obtained using phosphorylase as a substrate. The purified catalytic subunit and AC complex of PP-2A gave somewhat steeper inhibition curves, and the activity of the PP-2A catalytic subunit on C subunit was apparently more resistant to okadaic acid than that on phosphorylase. This difference is probably an artifact attributable to higher concentrations of the purified subunit required to achieve efficient C subunit dephosphorylation (see Fig. 7, below). Using phosphorylase as substrate, the recombinant PP-1 was clearly sensitive to inhibitor-2, but neither purified PP-2A nor the S49 cell activity showed any sensitivity to this inhibitor (data not shown).

The okadaic acid sensitivity of the reaction of PP-1 on C subunit could not be tested in the experiment of Fig. 5 because, as shown in Fig. 6, lanes c–e, recombinant PP-1 had no detectable activity on native C subunit. In the presence of 3 m urea, however, PP-1 was able to remove the phosphate from Thr-197 of C subunit (Fig. 6, lanes f–h). Neither potato acid phosphatase nor calf intestinal alkaline phosphatase were able to dephosphorylate C subunit in the presence or absence of urea.3

Fig. 7 compares the activities of PP-1, PP-2A, and the crude and partially purified S49 cell preparations on C subunit. The phosphatase concentrations were all expressed in terms of activity against glycogen phosphorylase. As noted above, PP-1 had no detectable activity on native C subunit. When normalized in this way, the S49 cell preparations were about 10 times more effective than the catalytic subunit of PP-2A as C subunit phosphatases. The AC complex of PP-2A appeared to be somewhat more effective on C subunit than the free catalytic subunit, but we had insufficient material to extend the curve to the higher concentrations required to achieve substantial C subunit dephosphorylation.

Phosphatase Accessibility of Thr-197 in the Native C Subunit Structure Is Limited under Physiological Conditions—Fig. 8 shows that low concentrations of urea enhance markedly the dephosphorylation of C subunit by S49 cell extracts. (The effect of urea was more difficult to assess with purified preparations of PP-2A or the S49 cell phosphatase because these preparations were more sensitive to urea-dependent inactivation.)

Fig. 8A shows that at a fixed concentration of phosphatase, urea concentrations as low as 0.5 m promoted dephosphorylation of C subunit at 30°C, where the native C subunit was not dephosphorylated significantly. Fig. 8B shows that the inclusion of 3 m urea shifted the phosphatase dose-response for dephosphorylation of C subunit at 30°C by about 2 orders of magnitude toward lower concentrations, while 1.5 m urea had a more moderate effect.

Several studies had suggested that the conformation of C subunit is sensitive to ionic strengths around the physiological range (19, 20), and C subunit stability is enhanced by ionic strengths above 100 mW (21). The experiment of Fig. 9 investigated the effect of salt concentration on C subunit dephosphorylation using the inactivation assay of Fig. 2. The rate of dephosphorylation was slowed progressively by almost 10-fold as the salt concentration was increased from 0 to 200 mM. Salt concentrations in this range had very little effect on the phosphatase activity against glycogen phosphorylase (data not shown).

The studies described to this point used recombinant C subunit that lacked the N-terminal myristoyl group that is present in the enzyme from mammalian tissues. To assess the effect of this group on the phosphatase reaction, the experiment of Fig. 10 compared dephosphorylation of nonmyristoylated C subunit with that of C subunit purified from bacteria that were co-expressing the yeast N-myristoyltransferase. The reactions again were monitored by measuring loss of kinase activity. With either 50 or 150 mM sodium chloride, dephosphorylation of the myristoylated C subunit was about 3-times slower than that of the nonmyristoylated subunit. The myristoylated form also appeared to be more resistant to phosphatase-independent

3 R. A. Steinberg, unpublished results.

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**Figure 5.** Okadaic acid sensitivities of the protein phosphatase activities of S49 cells are intermediate between those of the catalytic subunits of PP-1 and PP-2A. An S49 cell extract (lane a), anion exchange-purified S49 cell protein phosphatase (C), purified PP-2A catalytic subunit (B), the AC complex of PP-2A (D), or the recombinant catalytic subunit of PP-1 (A) was incubated with either glycogen phosphorylase a (A) or recombinant C subunit (B) for 2.5 h at 37°C in the absence or presence of okadaic acid at various concentrations. Phosphatase activity was monitored for A by loss of phosphorylase activity ("Experimental Procedures") and for B by conversion of C subunit to the faster migrating, Thr-197 dephosphorylated form (Fig. 1). For the reactions of A, phosphorylase was at 1.5 mg/ml, and phosphatase preparations were at about 1.3 mg/ml, 0.3 mg/ml, 0.3 μg/ml, 0.5 mg/ml, and 0.4 μg/ml, respectively, for the S49 extract, partially purified S49 phosphatase, PP-2A catalytic subunit, AC complex, and PP-1. For the reactions of B, C subunit was at 0.2 mg/ml, and phosphatases were at about 1.3 mg/ml, 2 mg/ml, 4 μg/ml, and 1 mg/ml, respectively, for the S49 extract, partially purified S49 phosphatase, PP-2A catalytic subunit, and AC complex. Because PP-1 had virtually no activity on native C subunit, its okadaic acid sensitivity with the C subunit substrate was not measured.

**Figure 6.** PP-1 can dephosphorylate Thr-197 of C subunit in the presence of urea, but not in its absence. C subunit at 0.25 mg/ml (lane a) was incubated for 3 h at 37°C with PP-1 in the absence (lanes c–e) or presence of 3 m urea (lanes f–h) and then analyzed by SDS-PAGE and silver-staining (as for Fig. 1). PP-1 concentrations were 6.3 (lanes c and f), 13 (lanes d and g), or 25 μg/ml (lanes e and h). PP-1 alone was diluted to give an amount identical to that from the 25 μg/ml samples (lane b).
Our results indicate that the Thr-197 phosphate of native, recombinant C subunit can be removed by a protein phosphatase activity found in extracts of S49 mouse lymphoma cells. This dephosphorylation could be monitored by either a mobility shift in SDS-PAGE or a reduction in protein kinase activity, and it resulted in loss of labeled phosphothreonine from C subunit autophosphorylated in the presence of \([\gamma-32P]ATP.\) Initial characterization suggested that the cellular activity was related to PP-1 and/or PP-2A. It was sensitive to fluoride ion and okadaic acid, which inhibit PP-1 and PP-2A, but resistant to the chelators EDTA and EGTA, which would have inhibited the Ca\(_{2+}\)-dependent PP-2B or the Mg\(_{2+}\)-dependent PP-2C (Fig. 1). Vanadate ions, which inhibit the dual-specific protein tyrosine phosphatases implicated in dephosphorylation of the MAP kinases (22), had no effect on the cellular phosphatase active against C subunit. Further analysis using okadaic acid at concentrations that can distinguish between PP-1 and PP-2A and inhibitor-2, which is selective for PP-1, suggested that the cellular activity was either a form of PP-2A or a closely related enzyme (Fig. 5, and data not shown). Consistent with these results, the catalytic subunit of PP-2A could dephosphorylate native C subunit but that of PP-1 showed no activity against this protein (Figs. 6 and 7). Fractionation of S49 cell extracts by anion-exchange chromatography resolved multiple peaks of protein phosphatase active against glycogen phosphorylase a,
all of which were inhibited by a concentration of okadaic acid that had no effect on PP-1. These probably represent different multimeric complexes of PP-2A (23). Only the later eluting peaks had significant activity against C subunit, suggesting that not all complexes of PP-2A were equally active on C subunit (Fig. 4). The okadaic acid-sensitivity of the S49 cell activity was more similar to that of an AC complex of PP-2A than to that of the PP-2A catalytic subunit (Fig. 5). Furthermore, when normalized for activity against phosphorylase α, the S49 cell phosphatase was about 10-fold more active against C subunit than was the purified catalytic subunit of PP-2A (Fig. 7). We conclude from these observations that the S49 cell activity is probably a multimeric complex of PP-2A (AC or ABC (23)), although we cannot rule out the possibility that it is a novel enzyme with properties very similar to PP-2A. The cell extracts used in these studies were gel filtered to remove an inhibitor (or inhibitors) of the phosphorylase phosphatase activity. The activity against C subunit was unaffected by gel filtration (data not shown), suggesting another distinction between the phosphorylase and C subunit phosphatase activities.

Treatment of C subunit with urea promoted dephosphorylation by the S49 cell activity (Fig. 8) and purified PP-2A (data not shown) and enabled the protein to be dephosphorylated by PP-1 (Fig. 4). On the other hand, neither potato acid phosphatase nor calf intestine alkaline phosphatase were able to dephosphorylate C subunit at Thr-197 with or without the chaperonic agent. These latter results might account for the widespread belief that the phosphate on Thr-197 was inaccessible to a chaperonic agent. These latter results might account for the widespread belief that the phosphate on Thr-197 was inaccessible to a chaperonic agent.

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The first published crystal structure of C subunit was a ternary complex with ATP and inhibitor peptide in which the phosphate on Thr-197 was closely associated with the functional groups of His-87, Arg-165, Lys-189, and Thr-195 (6). Structures of the apoenzyme and binary complexes of C subunit with an iodinated inhibitor peptide now have been reported and differ from the original structure primarily by rotation of the smaller of the protein’s two lobes to give a more open conformation around the catalytic cleft (24, 25). Where His-87 interacts with the Thr-197 phosphate in the closed ternary complex conformation, it is nearly 6 Å away in the open conformation. We suspect that this opening of the cleft and disruption of the interaction between His-87 and the Thr-197 phosphate is responsible for making the phosphate accessible to removal by PP-2A. The inhibitory effect of elevated salt on C subunit dephosphorylation (Fig. 9), then, would suggest that salt concentrations in the physiological range shift the dynamic equilibrium toward the closed conformation. Treatment of C subunit with concentrations of urea below 2 M had relatively small effects on the subsequent activity of C subunit, while increasing concentrations between 2 and 4 M led progressively to irreversible inactivation. The enhanced dephosphorylation of C subunit at low concentrations of urea (Fig. 8), therefore, probably reflected a loosening or opening of the structure around Thr-197 rather than complete denaturation of the protein. A similar enhancement without urea was observed in a C subunit preparation that had been partially inactivated by storage in buffer without phosphatase (see “Experimental Procedures”). We attempted to assess the effect of Mg<sup>2+</sup>-ATP or Mg<sup>2+</sup>-ATP-S<sub>4</sub> on the dephosphorylation reaction, since these compounds favor the closed conformation of C subunit, but, unfortunately, these compounds were also strong inhibitors of PP-2A (data not shown).

We conclude that native C subunit can assume a conformation in which its critical phosphate on Thr-197 is susceptible to dephosphorylation by PP-2A and that this susceptibility is reduced by physiological ionic strength and the presence of an amino-terminal myristoyl group, two factors reported to stabilize C subunit structure (21, 25). The reaction in vitro on native, myristoylated C subunit under near physiological conditions of temperature, pH, and ionic strength was sufficiently slow (e.g. t<sub>0.5</sub> > 2 h in the experiment of Fig. 10) to question whether there is significant turnover of the Thr-197 phosphate in vivo. On the other hand, if dephosphorylation rates are linear with phosphate concentration and all of the active phosphate in our extracts is active in intact cells, the intracellular t<sub>0.5</sub> for turnover of the Thr-197 phosphate could be as short as about 20 min.

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Dephosphorylation of Catalytic Subunit of cAMP-dependent Protein Kinase at Thr-197 by a Cellular Protein Phosphatase and by Purified Protein Phosphatase-2A
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