On the Mechanism of Regulation of Type I Phosphoprotein Phosphatase from Bovine Heart

REGULATION BY A NOVEL INTRACYCLIC ACTIVATION-DEACTIVATION MECHANISM VIA TRANSIENT PHOSPHORYLATION OF THE REGULATORY SUBUNIT BY PHOSPHATASE-1 KINASE (FA)*

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Adenosine 5’-(γ-thio)triphosphate (ATPγS) can substitute for ATP in the activation of the ATP-Mg2+-dependent form of bovine heart type I protein phosphatase (M₀ = 75,000) catalyzed by phosphatase-1 kinase (FA). ATPγS activates the enzyme to a lower level than ATP, but it phosphorylates the regulatory (R)-subunit to a much higher extent. An [35S]phosphatase-1 ([35S]E-P) has been isolated, identified, and shown to be a key intermediate in the activation reaction. Treatment of [35S]E-P with dimethyl suberimidate results in cross-linking of the M₀ = 34,000 [35S]R-subunit with the M₀ = 40,000 catalytic (C)-subunit to form a M₀ = 75,000 species, indicating that phosphorylation is not accompanied by dissociation of the holoenzyme. The catalytically active form (E₀) is not the phosphorylated enzyme intermediate. Instead, E₀ is directly produced from the intermediate by a Mg2+-dependent, intramolecular autodephosphorylation reaction. The isolated E₀ derived from [35S]E-P or from ATP-activated phosphatase-1 has the same half-life (23 min at 30°C). It spontaneously deactivates, via an intramolecular process, to a resting state (Eₚ) which can be fully reactivated by Fₐ·ATP·Mg2+. The deactivation of E₀ can be accelerated by chelators, PP₁ > ATP. Mg2⁺ blocks the PP₁ effect. Limited trypsinization selectively digests the R-subunit and the resulting C-subunit is Mg2+-dependent. Based on the present data, a novel intracyclic activation-deactivation mechanism via transient phosphorylation of the R-subunit is proposed for regulation of phosphatase-1.

E₀ (C) ATP Mg2+ ADP → E-P (C·Mg2+) → Eₚ

Type I phosphoprotein phosphatase (phosphatase-1) was first designated by Cohen (1) as an operational term for a rabbit skeletal muscle phosphatase activity that is sensitive to inhibition by heat-stable proteins, inhibitor-1 (I-1) and inhibitor-2 (I-2), and exhibits high specificity towards the δ-subunit of phosphorylase kinase (1–3). It turns out that the holoenzyme form of phosphatase-1 requires the simultaneous presence of Mg2⁺, ATP, and a protein activator, termed Fₐ, for expression of its activity (4–11). The holoenzyme form was discovered and designated by Merlevede and co-workers (10) as ATP·Mg2+-dependent phosphoprotein phosphatase or Fₐ·M, where Fₐ and M represents catalytic component and modulator protein (i.e. inhibitor-2), respectively (4–10). Fₐ, which was found to exhibit a protein kinase activity towards glycogen synthase (4, 5), was subsequently shown to be similar to glycogen synthase kinase 3 (12).

Most studies done on phosphatase-1 have dealt with the various forms of the enzyme isolated from rabbit skeletal muscle (1–20). These include Fₐ·ATP·Mg2+-activatable and -nonactivatable forms of M₀ = 70,000-140,000 and M₀ = 30,000-40,000 respectively. The latter may represent free catalytic (C)-subunit or its degradative product. Several reports indicate that the M₀ = 70,000 (70K) form may exist as a C-subunit (38K): I-2 (31K) complex (13–16). However, due to the unavailability of highly purified holoenzyme, the subunit composition of phosphatase-1 has not been well-established. It has been suggested that the C-subunit may be a 60–70K protein (4–10, 17).

The activity of phosphatase-1, either the Fₐ·ATP·Mg2+-activatable or -nonactivatable forms, can be detected in the presence of Mn2⁺ alone (6–11, 15, 16, 23), and the early studies on the enzyme dealt with this Mn2+-activated activity (1–3, 21–24). Since the discovery that the holoenzyme is under regulation by a protein kinase activity (4–5), several studies have indicated that phosphorylation of the I-2 component at a Thr residue (12, 18) may play a role in the activation process (13–18). Taking advantage of the fact that thiophosphoproteins are generally more resistant to phosphatase activity than their normal counterparts (25, 26), we have dissected the activation process of phosphatase-1 by using adenosine 5’-(γ-thio)triphosphate (ATPγS) in place of ATP as an activator. As a result, we have successfully isolated two key intermediates in the activation reaction: an inactive 35S-labeled thiophosphorylated enzyme and an activated nonthio-
phosphorylated enzyme. Based on the present data, the subunit composition and activation mechanism which involves cyclic phosphorylation-dephosphorylation of the regulatory (R-) subunit are proposed for the cardiac muscle type I phosphoprotein phosphatase.

**EXPERIMENTAL PROCEDURES**

**Materials—**[γ-32P]ATP and [35S]ATP were from Amer sham Corp. and New England Nuclear, respectively. Nonlabeled ATP-S was from Boehringer Mannheim. Dimethyl suberimidate was from Pierce Chemical Co. TPC-treated trypsin and soybean trypsin inhibitor were from Worthington.

Enzyme phosphorylation on 10 or 12.5% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out according to Laemmli (27). Proteins in samples were precipitated with 12.5% trichloroacetic acid prior to electrophoresis. Gels were stained with Coomassie Brilliant Blue R-250.

The subunits of phosphatase-1 were quantitated by densitometric analysis of Coomassie Blue-stained gels, using an EC-980 Densitometer with a Hewlett-Packard 3380 Reporting Integrator. The color values of different subunits were assumed to be proportional to their molecular weights as determined by SDS-PAGE.

**Isolation and Proteins—**Phosphatase-1 was purified from bovine cardiac muscle by a procedure involving (NH4)2SO4 fractionation, DEAE-cellulose, Sarcophany S-200, and polylysine-Sepharose chromatographies (28) similar to that described by Yang et al (4). SDS-PAGE analysis of several preparations indicated that the sum of the R-subunit (34K) and the C-subunit (40K) represented about 10-35% of the total proteins in these preparations, as estimated by densitometry scanning of the gels. Phosphatase-1 kinase (F3) was purified from the same tissue by a procedure involving DEAE-cellulose, Sarcophany S-200, and Red A Matrix Gel (Amicon) chromatographies (29). [32P]Phosphorylase a was prepared by phosphorylation of crystalline phosphorylase b from rabbit skeletal muscle with phosphorylase kinase and [γ-32P]ATP as previously described (30, 31). Inhibitors-1 (3) and -2 (33) were purified from rabbit skeletal muscle.

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**RESULTS**

**Time Course of Activation by ATP and ATP-S—**As shown in Fig. 1, the activation by ATP does not parallel the incorporation of 32P into the R-subunit (Fig. 1a) while that by ATP-S does (Fig. 1b). Furthermore, ATP-S activates the phosphatase activity at a much slower rate (about 5-7% of that of ATP) and to a much lower extent (above 20-25% of that of ATP) but is accompanied with a much higher level of phosphorylation (incorporation of 32P into the R-subunit is at least 30-fold higher than 32P). Addition of excess EDTA to block the kinase reaction results in a rapid decrease of both the ATP-S and the ATP-activated activities without affecting the level of either the bound 32S or the bound 32P.

The data are interpreted to mean that both the rate and extent of activation are determined by the turnover rate of the phospho(thio)phosphate group on the R-subunit but not by the extent of its phosphorylation (or thionophosphorylation) as detected at selected time points during the course of the activation reactions. In other words, it is the dephosphorylation step, rather than the phosphorylation step which is responsible for generation of the phosphorylase phosphatase activity. On the basis of this concept, the differences between the activation-phosphorylation curves presented in Fig. 1, a and b, can be readily explained by assuming that ATP-S is a much poorer substrate for the F3 kinase activity. Therefore, the rate of phosphorylation of the R-subunit is much faster.
than that of thiophosphorylation. In the case of ATP activation, the rate of dephosphorylation is faster than that of phosphorylation and the reverse is true for the ATPγS activation reaction. The observed EDTA effects suggest that the presence of Mg2+ is essential for dephosphorylation of the phospho-enzymic intermediate and/or for maintenance of the phosphatase activity generated. The fact that the ATPγS- and the ATP-activated activities exhibit identical response to phosphorylation. The resultant [35S]E-P was then isolated by precipitation and extensive dialysis in the presence of Mg2+. The results indicate that the inhibitory activity is associated with the regulatory subunit.

Isolation of [35S]Phosphatase-1 —The observations that: (a) the level of [35S] incorporation is high (b) the thiono-phosphorylation is stable in the presence of EDTA (Fig. 1b) indicate that it is feasible to isolate the thiono-phosphoenzyme intermediate for detail analysis of its properties. To do this, phosphatase-1 was incubated for a prolonged period of time with [35S]ATPγS, Mg2+, and Fx, to insure maximum thiono-phosphorylation. The resultant [35S]E-P was then isolated by repetitive (NH4)2SO4 precipitation and extensive dialysis in the presence of 0.1 mM EDTA (details are presented under “Experimental Procedures”). No significant release of [35S] was observed after stored at ~20 °C for at least 6 months.

Molecular Property of the Isolated [35S]E-P —As shown in Fig. 2a, the isolated [35S]E-P is eluted from Sephacryl S-200 as a single peak of phosphorylase phosphatase activity corresponding to an apparent Mr = 89,000, which is identical to that of the native phosphatase-1. The elution profile of the phosphatase activity coincides with that of: (a) [35S]-protein and (b) the heat-stable inhibitory activity associated with phosphatase-1. In order to identify the thiono-phosphorylated component(s), SDS-PAGE of the active fractions was carried out. As shown in Fig. 2b (right panel), protein staining bands corresponding to Mr = 40,000 and 34,000, respectively, are enriched in the active peak fractions. Autoradiography demonstrates that only the 34K staining band is radioactive (Fig. 2b, left panel). An identical, unstained gel was sliced into 1-mm sections and protein in each section was extracted and assayed for inhibitory activity towards the Fx-ATP-Mg2+-activated native phosphatase-1. The results indicate that the inhibitory activity is associated with the Mr = 34,000 species (data not shown). Densitometry tracing of the Coomassie Blue-stained gels reveals that the molar ratio of the 40K and the 34K species is about 1:1. In a separate study, we have purified the phosphatase to near homogeneity by an improved purification procedure.2 SDS-PAGE analysis of the enzyme preparation shows only two major protein staining bands of Mr = 40,000–41,000 and 34,000–35,000, respectively, in a molar ratio of about 1:1. The two species account for more than 85% of the proteins detectable in the enzyme preparation. These and results by others (16) have led us to conclude that the 40K protein represents the C-subunit of the phosphatase.

If thiono-phosphorylation of the 34K R-subunit does not result in dissociation from the 40K C-subunit, then, these two components should be able to be cross-linked by dimethyl suberimidate to form a 75K species. As shown in Fig. 3 (lower panel), incubation of [35S]phosphatase-1 with increasing concentrations of dimethyl suberimidate causes a progressive decrease in the 34K and the 40K staining bands with a concomitant increase of a 75K species on the SDS-PAGE. Autoradiography of the same gel shows a decrease of a 34K radioactive band and appearance of a major doublet radioactive band corresponding to Mr = 75,000 (Fig. 3, upper panel).

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FIG. 3. Cross-linking of the regulatory and the catalytic subunits of $[^{35}S]$phosphatase-I. The cross-linking reaction was carried out in a volume of 200 µl containing 0.2 M triethanolamine-HCl, pH 8.5, 25% glycerol, and $[^{35}S]$phosphatase-1 (50 nM bound $^{35}S$, 45 µg/ml protein) in the absence (lanes 1 and 8) and presence of 1.25 (lane 2), 2.5 (lane 3), 4 (lane 4), 10 (lane 5), and 20 mM (lanes 6 and 7) dimethyl suberimidate. After incubating at 30 °C for 30 min (lanes 1–6) or for 60 min (lanes 7 and 8), reactions were terminated by the addition of 500 µl of 10% trichloroacetic acid. Proteins were collected by centrifugation and subjected to SDS-PAGE, the gel was stained, dried, and autoradiographed. The upper and lower panels represent the autoradiogram and protein staining patterns, respectively. HS, high molecular weight protein standards; myosin (200K), P-galactosidase (116K), phosphorylase b (92.5K), BSA (66.5K), and ovalbumin (45K). Low molecular weight protein standards (LS); phosphorylase b, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme.

Conversion of the Isolated $[^{35}S]$E-P to a Catalytically Active Form (E2)—As predicted by the data shown in Fig. 1, the isolated $[^{35}S]$E-P exhibits little phosphatase activity. Among several compounds examined, only Mg$^{2+}$ and Mn$^{2+}$ are found to be effective in stimulating the release of $^{35}S$ with concomitant generation of the phosphatase activity (Table I). The Mg$^{2+}$ activating effects are not significantly affected by each of these compounds examined except when both F$_{A}$ and ATP are present. In such a condition, the Mg$^{2+}$ activating effect in generation of phosphatase activity is stimulated by FA plus ATP to a much higher extent than that on $^{35}S$ release. The phenomenon is consistent with the interpretation that the nonthiophosphorylated enzyme molecules, including those newly generated from $[^{35}S]$E-P, have been activated by the ATP-supported mechanism. The observation that phosphatase a shows a slightly stimulatory effect is of interest. This
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**TABLE I**

| Addition                  | Radioactivity Release (pM/mg) | Phosphoprotein Phosphatase Activity (U/mg) |
|---------------------------|------------------------------|-------------------------------------------|
| None                      | 77                           | 1                                         |
| MgCl₂                     | 670                          | 67                                        |
| MnCl₂                     | 1140                         | 68                                        |
| MgCl₂, ATP                | 760                          | 79                                        |
| FA                        | 110                          | 0.6                                       |
| FA, ATP, MgCl₂            | 830                          | 140                                       |
| MgCl₂, phosphorylase a    | 730                          | 69                                        |
| MgCl₂, phosphorylase b    | 740                          | 70                                        |

**Fig. 4.** Effects of Mg²⁺ concentrations on the dephosphorylation and activation of [³⁵S]phosphatase-1. [³⁵S]Phosphatase-1 (40 nM bound ³⁵S, 20 μg/ml protein) was incubated at 30°C in a volume of 500 µl containing 50 mM Tris-HCl, pH 7.4, 0.2 mg/ml BSA and various concentrations of MgCl₂ as indicated. Aliquots were removed for determining ³⁵S release and spontaneous phosphatase activity by the Direct Assay as described in the text. The arrow indicates the time at which EDTA was added to the preincubation mixtures so that its concentration was 1.8 mM in excess of Mg²⁺.

**Fig. 5.** Time course of dephosphorylation and activation of [³⁵S]phosphatase-1. [³⁵S]Phosphatase-1 (40 nM bound ³⁵S, 20 μg/ml protein) was incubated at 30°C in a volume of 500 µl containing 50 mM Tris-HCl, pH 7.4, 0.2 mg/ml BSA without or with the addition of 5 mM MgCl₂, 1 mM MnCl₂, 50 µM ATP, 0.2 units/ml FA, 0.1 µM phosphorylase b and nonlabeled phosphorylase a, separately or in combination as indicated. Aliquots were removed for determining ³⁵S release or for measuring the spontaneous phosphatase activity by the Direct Assay as described in the text.

**Fig. 6.** Effects of [³⁵S]phosphatase-1 concentrations on the rate of dephosphorylation. Incubation was the same as that described in the legend to Fig. 5 except that 27.6 nM (□) and 138 nM (■) of [³⁵S]phosphatase-1 were used. The inset indicates a semi-log plot of the same data.

indicates that the catalytic site responsible for autodethiophosphorylation (designated as Site A) differs from that designated as Site S for the genuine substrate, phosphorylase a. Therefore, phosphorylase a fails to act as a competitive inhibitor with respect to the thiophospho group on the R-subunit. Table I shows that Mn²⁺ and Mg²⁺ generate similar levels of phosphoprotein phosphatase activity but Mn²⁺ is more effective than Mg²⁺ in stimulating ³⁵S release.

As shown in Fig. 4, both the activation and dephosphorylation of [³⁵S]E-P increase in a parallel manner with the increase of Mg²⁺ concentrations. Both reach a maximum at about 5 mM Mg²⁺. Higher concentrations of Mg²⁺ result in a slight decrease in the activating effect. The concentration of Mg²⁺ for half-maximum activation-dephosphorylation is estimated as 0.6 mM.

As shown in Fig. 5, incubation of [³⁵S]E-P with 5 mM MgCl₂ at 30°C results in a time-dependent increase of the phosphatase activity. These do not occur in the absence of added Mg²⁺. Addition of excess EDTA to chelate Mg²⁺ results in termination of ³⁵S release and a decrease of the phosphatase activity. The decrease in activity is not due to denaturation of the activated enzyme by EDTA since it can be fully reactivated by incubating with FA-ATP-Mg²⁺ (Fig. 5). These data collectively suggest that the activated phosphatase-1 (Eₐ) has a finite life span and it spontaneously reverts to a resting state.
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Fig. 7. Spontaneous deactivation of the activated forms of phosphatase-1 derived from the Mg$^{2+}$-activated $[^{35}S]$enzyme and the F$\alpha$·ATP·Mg$^{2+}$-activated native enzyme. The activated phosphatase-1 preparations, which had been separated from low molecular effectors, were prepared as described in the text. The preparation (containing 38 nM bound $[^{35}S]$) derived from [$$]^{35}S$$]enzyme (O, D) and that derived from native enzyme (O, D) were separately incubated with 50 mM Tris·HCl, pH 7.4, 1.2 mg/ml BSA at 30°C. At the indicated times, aliquots were removed from each incubation mixture for measuring the spontaneous activity (O, D) and the F$\alpha$-ATP·Mg$^{2+}$-activated activity (O, D) by Direct and Preincubation Assays, respectively. The activities were expressed as per cent of those obtained at zero time incubation. At zero time, 0, 0, and 0 represent 4.4, 24.1, 2.1, and 2.2 units/ml of phosphatase activity, respectively, in the incubation mixtures. The inset indicates a semilog plot of the spontaneous activity.

state (E$\beta$), which can be again reactivated by the F$\alpha$ kinase reaction. Fig. 5 also shows that the phosphatase activity measured with F$\alpha$·ATP·Mg$^{2+}$ is progressively increased with the increase of $[^{35}S]$ release, reflecting the increase of E$\beta$ concentration available for reactivation.

Fig. 6 shows that, in the presence of 5 mM Mg$^{2+}$, the rate of $[^{35}S]$ release measured with 20 nM $[^{35}S]$E-P is identical to that with 100 nM $[^{35}S]$E-P. The rate is also similar to that measured with 40 nM $[^{35}S]$E-P as indicated in Fig. 5. The data indicate that the dethiophosphorylation-activation process is an intramolecular rather than an intermolecular event. The inset in Fig. 6 shows that $[^{35}S]$ release follows first order kinetics. The apparent rate constant, $k_{obs}$, is estimated as 0.013 min$^{-1}$ which is equivalent to a half-life ($t_{1/2}$) of 53 min.

Isolation of the Activated Form (E$\beta$) Derived from $[^{35}S]$E-P and from Native Phosphatase-1 Activated by ATP—As shown in Table II, at 1 mM concentration EDTA and EGTA show little effects on accelerating the deactivation of E$\beta$ derived from either $[^{35}S]$E-P or the native enzyme. In contrast, PP is more effective than ATP. The accelerating effects are not due to irreversible inactivation of the enzyme, since both the ATP- or the PP-treated enzymes can be fully reactivated by incubating with F$\alpha$·ATP·Mg$^{2+}$. Table II also shows that the E$\beta$ derived from the native enzyme is slightly more sensitive to both ATP and PP, than that derived from $[^{35}S]$E-P. The reason for this difference is not understood.

Fig. 8 shows that the accelerating effect of PP, on deactivation of E$\beta$, derived from native phosphatase-1 is dependent on PP concentration. Moreover, the effect of PP is cancelled by the presence of excess Mg$^{2+}$ (5 mM). The data are consistent with the interpretation that E$\beta$ contains an essential cofactor, Mg$^{2+}$, in the catalytic site. PP and ATP, due to their structural similarity to the phosphoprotein substrate and their chelating ability, can readily enter catalytic sites to remove Mg$^{2+}$ and, thus, can accelerate the deactivation of E$\beta$. The presence of excess Mg$^{2+}$ abolishes the PP, chelating function and, therefore, can cancel its effect. The ineffectiveness of EDTA and EGTA may be due to the fact that they bear no structural similarity to the substrate and, thus, cannot readily enter catalytic sites to exert their chelating function.

Limited Trypsinization of Phosphatase-1—As shown in Fig. 9, native phosphatase-1 shows little activity when measured with Mg$^{2+}$ alone. Incubation of the enzyme with 2 µg/ml of trypsin at 4°C in the absence of added divalent cation results in a rapid increase of a Mg$^{2+}$-activated activity to the level of the activity measured with the simultaneous presence of F$\alpha$·ATP·Mg$^{2+}$. This is accompanied with the disappearance of the 54K R-subunit (Fig. 9, inset). Mn$^{2+}$ (1 mM) or Co$^{2+}$ (1 mM) can substitute for Mg$^{2+}$ in the activation of the trypsinized enzyme and Co$^{2+}$ is slightly more effective. The activity measured with Mn$^{2+}$ is about 1-fold higher than that of Mg$^{2+}$. 
Effects of chelators on the activated forms of phosphatase-1 derived from native or $^{35}$S-enzyme

The activated phosphatase-1 preparations, which had been isolated from low molecular weight effectors, were incubated at 30 °C in a mixture containing 50 mM Tris·HCl, pH 7.4, 0.2 mg/ml BSA without and with the addition of 1 mM EDTA, EGTA, ATP, and PP, as indicated. After 10 min, aliquots were removed for measuring the spontaneous and the $F_A$-ATP·Mg$^{2+}$-activated activities by Direct and Preincubation Assay, respectively. The parenthesis indicates the percent activity of those without the addition of a chelator.

| Addition | Native enzyme | $^{35}$S-enzyme |
|----------|---------------|-----------------|
|          | Spontaneous   | $F_A$-ATP·Mg$^{2+}$ | Spontaneous | $F_A$-ATP·Mg$^{2+}$ |
| None     | (100) 6.1     | (100) 7.3       | (100) 1.2   | (100) 7.34 |
| EDTA     | (92) 5.6      | (97) 7.0        | (93) 1.1    | (101) 7.3  |
| EGTA     | (95) 5.8      | (100) 7.2       | (92) 1.1    | (103) 7.4  |
| ATP      | (79) 4.8      | (98) 7.1        | (87) 1.0    | (99) 7.3   |
| PP       | (52) 3.2      | (94) 6.8        | (60) 0.72   | (101) 7.4  |

**DISCUSSION**

We have found that ATPγ-S can substitute for ATP in the activation of phosphatase-1. A $^{35}$S-labeled thiophosphorylated enzyme has been isolated, identified, and shown to be a key intermediate in the activation processes. We further demonstrate that the enzymatically active form of phosphatase-1 is not the phosphorylated enzyme intermediate. Instead, it is directly produced from the intermediate by a Mg$^{2+}$-dependent autodephosphorylation reaction. The data do not support the hypothesis that the phosphorylated enzyme is the catalytically active form as suggested by several laboratories (14-18). The present studies demonstrate that the autodephosphorylation of the intermediate is an intramolecular event rather than a process catalyzed by a separate activated phosphatase as suggested by others (13, 14, 16, 17). The availability of isolated $^{35}$S-labeled thiophosphorylated enzyme in non-denatured form has provided a powerful means for investigating the molecular properties of phosphatase-1. These experiments have provided clear cut evidence that: (a) phosphatase-1 is a heterodimer of 75K consisting of a C-subunit of 40K and an inhibitory R-subunit of 34K and (b) in the process of activation, the phosphorylated R-subunit remains in association with the C-subunit. The data are in agreement with those by Villa-Moruzzi et al. (16) and Jurgensen et al. (17), but differ from those by Hemmings et al. (13, 14) who reported that phosphorylation of the I-2 component of a reconstituted phosphatase-1 resulted in its dissociation from the catalytic component, leading to expression of the enzymatic activity. The dissociation mechanism was further supported by the observation that cross-linking of the I-2 with the catalytic component.
that ATPγS could not replace ATP in the activation of either skeletal muscle, despite the fact that it could thiophosphorylate the 1-2 component (13, 14). Furthermore, Hemmings et al. (13) reported that thiophosphorylation prevented the enzyme from being activated by FA.ATP.Mg+.

In contrast to the present results, several studies indicated that ATPγS could not replace ATP in the activation of either native (4, 7, 10) or reconstituted (13, 14) phosphatase-1 from skeletal muscle, despite the fact that it could thiophosphorylate the 1-2 component (13, 14). Furthermore, Hemmings et al. (13) reported that thiophosphorylation prevented the enzyme from being activated by Fα-ATP-Mg2+. The reason for these discrepancies is not clear.

The R-subunit of phosphatase-1 has generally been referred to as I-2 (1-5, 12-16, 18-20, 23) or modulator protein (6-10, 17), reflecting the current concept that it may be a free regulator protein in vivo, similar to I-1. This concept has stemmed from historical ground. Both I-1 and I-2 had been discovered (37-41), extensively studied (1-3, 10, 23), and considered as free regulatory proteins in tissue long before any knowledge concerning the molecular composition of the phosphatase-1 holoenzyme was known. However, there is no experimental evidence indicating the I-2 exists as a free form in vivo. On the other hand, several lines of evidence indicate that it does exist as an integral subunit of the ATP-Mg2+-dependent phosphatase. (a) We have found that I-2 co-purifies with the ATP-Mg2+-dependent phosphatase from bovine heart throughout our purification procedures (28). (b) Yang et al. (9) have reported that it co-purifies with the phosphatase from rabbit skeletal muscle throughout their purification procedures. (c) Ballou et al. (15) have found that the molar ratio between I-2 and C-subunit in the purified phosphatase-1 is 1:1. (d) Resink et al. (14) have estimated the in vivo concentrations of I-2 and C-subunit in rabbit skeletal muscle and concluded that the molar ratio between them is approximately 1:1. The fact that the I-2, but not C-subunit, is extremely sensitive to proteolytic attack, also works against the free regulatory protein concept. We propose that in vivo, I-2 exists as an integral subunit of phosphatase-1 holoenzyme. Therefore, we designate it as "R-subunit," replacing the conventional terms "I-2" and "modulator protein."

Based on the present data, a model for activation of phosphatase-1 from cardiac muscle is presented in Fig. 10. In the resting state, phosphatase-1 is inactive (Ei). Upon phosphorylation of the R-subunit, the enzyme changes its conformation, leading to the formation of active Site A, which is specific for the phospho group on the R-subunit of the same enzyme molecule (E-P). Site A requires Mg2+ as a cofactor for expressing its catalytic activity \( \frac{E \cdot Mg^{2+}}{P} \). The Mg2+ could be derived directly from the Mg2+-ATP complex or in rapid equilibrium with free Mg2+ in the medium. Intramolecular autophosphorylation of the R-subunit with concomitant insertion of Mg2+ results in the transformation of Site A into Site S on the C-subunit, which is specific for the extramolecular substrate, phosphorylase a. The activated enzyme (Ea) has a finite life span. It spontaneously reverts to Ei. During this intramolecular event, Mg2+ is released and Ei is then ready for the next round of activation.

In this model, Mg2+ plays a central role: (a) it forms a Mg2+-ATP complex to serve as a substrate for phosphatase-1 kinase and (b) it serves as a cofactor for Sites A and S. The model indicates that continued hydrolysis of ATP is required for the maintenance of the activated form, consistent with the observation by Jurgensen et al. (17) that, in activation of phosphatase-1, Fα exhibits an ATPase activity.

**Fig. 9. Limited trypsinization of phosphatase-1.** Incubation was carried out at 4 °C in a mixture containing 50 mM Tris·HCl, pH 7.4, 20% glycerol, and 0.29 mg/ml phosphatase-1 in the absence (□, ■) or presence (○, ●, ◦) of 2 μg/ml trypsin. At the indicated time, trypsin inhibitor (25-fold excess) was added to terminate the reaction. Aliquots were then removed for measuring the phosphatase activity by the Preincubation Assay in the absence (○, 9, 0) or presence (●, 8, 8) of FA, ATP plus MgCl2 (■, ◦) as described in the text. Aliquots of 100 μl were also withdrawn for SDS-PAGE analysis. The bars on the left side of the inset indicate the position of protein standards: phosphorylase a (92.5K), BSA (67K), ovalbumin (45K), carbonic anhydrase (31K), and trypsin inhibitor (21.5K), respectively.

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The relative rates among the three steps in the activation-deactivation cycle, $E_R \xrightarrow{k_1} E_P \xrightarrow{k_2} E_0 \xrightarrow{k_3} E_R$, are estimated as follows. For the ATP- and ATPγS-supported processes, the orders would be $k_2 \geq k_1 \geq k_3$ (0.030 min⁻¹) and $k_2 > k_3 (0.030$ min⁻¹) > $k_2 (0.013$ min⁻¹), respectively, where $k_1$ (ATP) > $k_1$ (ATPγS). $k_2$ (ATP) > $k_2$ (ATPγS), and $k_3$ (ATPγS). These are consistent with the characteristic time courses for the ATP- and ATPγS-supported activations shown in Fig. 1, a and b, respectively.

Considerations concerning the possible conformational changes of the two subunits during the activation-deactivation cycle are presented as follows. It is assumed that the C- and R-subunit in $E_R$ are in the T (tight) and R (relaxed) states, respectively (Fig. 10). The effects of phosphorylation would be in transforming the R-subunit into a partially T state which allows the formation of Site A on the C-subunit. The energy released from autodephosphorylation reaction drives the R-subunit into a transient-stable T state and the active site is thus uncovered ($E_A^* \xrightarrow{k_2} E_T \xrightarrow{k_3} E_{T*}$). The transition of the R-subunit from the T to the R state would result in a decrease of its physical interaction with the C-subunit and vice versa. The postulation is consistent with the observation by Villa-Moruzzi et al. (16) that the phosphorylated form of phosphatase-1 is more susceptible than the nonphosphorylated form to dissociation by polyvalent SI chromatography at pH 7.0. It also offers an explanation for the observation that phosphorylation caused dissociation of reconstituted phosphatase-1 (13, 14). An alternate model is that the conformation state of Site A is identical to that of Site S. The failure of phosphorylase $a$ to inhibit autodephosphorylation of $E_P$ may be explained by assuming that the active site has been occupied by the phospho-group on the R-subunit and, therefore, is unaccessible to extramolecular phosphoprotein substrate. The third model is that the C-subunit exists only in the R state and does not change its conformation during the activation-deactivation cycle. In such a model, the free active site is in a relaxed state ($E_{R*}$). The energy released from the phosphorylation-autodephosphorylation cycle drives the R-subunit into a transient-stable T state and the active site is thus uncovered ($E_A^* \xrightarrow{k_2} E_T \xrightarrow{k_3} E_{T*}$). The spontaneous reversion of the R-subunit from the T to the R state results in again covering up the active site ($E_A^*$). We favor the first model (Fig. 10), since it represents the most general case. It offers a plausible explanation for the relationship between the phosphorylation-dephosphorylation cycle of the R-subunit and activation-deactivation of the phosphatase activity. It also explains the facts that: (a) ATP hydrolysis is required for each activation-deactivation cycle, (b) the free C-subunit is in an activated state, and (c) the free R-subunit is spontaneous inhibitory. The present model indicates that formation of the active site is a consequence of R-subunit dislocation. Therefore, added free R-subunit (I-2) could readily occupy the active site. The postulation offers an explanation for the I-2 inhibition on the $E_{AA}$-ATP-Mg²⁺-activated activity and is consistent with the kinetic studies that I-2 acts as a competitive inhibitor with respect to phosphorylase $a$ (42).

The present data (Fig. 9) and results by others (6, 10, 14-15) indicate that the R-, but not the C-subunit, is extremely sensitive to proteolysis. Therefore, proteolytic damage of the R-subunit is likely to occur during purification manipulation. Minor damage on the phosphorylation site or other functional parts of the R-subunit may not result in dissociation of the
holoenzyme or reduction of its apparent size but may impair the regulatory function of the R-subunit. If proteolysis occurs in $E_r$, the resulting enzyme would exhibit an increase in sensitivity to activation by divalent cation alone, since the C-subunit has been transformed to the R state without incorporation of the cofactor. These explain the general observations that the sensitivities of phosphatase-1 holoenzyme to holoenzyme or reduction of its apparent size but may impair through preparation of phosphatase-1 allowed the recovery progressively during purification. Addition of protease inhibitors throughout preparation of phosphatase-1 allowed the recovery of an enzyme preparation that was poorly activated by Mn$^{2+}$ (15). A similar phenomenon has also been observed by others (6, 10).

The R-subunit in $E_r$ is responsible for transformation of the C-subunit from the R to T state and for expelling Mn$^{2+}$ from the active site. Therefore, proteolytic damage of the R-subunit in $E_r$ will result in locking the enzyme in its catalytically active form indefinitely. Various molecular forms of phosphatase-1 ($M_r = 30,000-260,000$) which are spontaneously active and insensitive to stimulation by Mn$^{2+}$, have been isolated from skeletal muscle by several laboratories (1-3, 10, 19, 20, 23). These may be derived from $E_r$ in which the R-subunit has been either dissociated or damaged by proteolytic attack. Villa-Moruzzi et al. (16) reported that the free C-subunit dissociated from the $F_A$, ATP-Mg$^{2+}$-activated phosphatase-1 ($E_r$) by polyanion SF chromatography was spontaneously active and slightly inhibited by Mn$^{2+}$ while that from the native, inactive enzyme ($E_r$) was Mn$^{2+}$-dependent, consistent with the present interpretations. The C-subunit derived from $E_r$ is much more stable than that from $E_r$ (16). Thus, it is reasonable to assume that the C-subunit (or its degradative products) derived from $E_r$ is more resistant than that from $E_r$ to harsh dissociation processes, such as precipitation by 80% ethanol at room temperature. Therefore, the low molecular forms of phosphatase-1 ($M_r = 30,000-35,000$) purified by procedures involving ethanol treatment may be derivatives of $E_r$ rather than $E_r$. Indeed, these enzyme preparations are generally spontaneously active and insensitive to stimulation by Mn$^{2+}$ (1-3, 13, 14, 18, 19).

The present data indicate that, if phosphorylation occurs at a single Thr residue, incorporation of either phosphate or thiophosphate into the R-subunit will never reach a 1:1 molar ratio, since the phosphorylation is transient. DePaoli-Roach et al. (18) reported that phosphorylation of the R-subunit is far below the 1:1 molar ratio, in agreement with our data. Hennings et al. (13), however, reported that thio phosphorylation stoichiometry approached 1 mol of $^{35}$S incorporated per mol of reconstituted enzyme. If so, phosphorylation of the enzyme might occur at multiple Thr residues.

The present studies indicate that metabolites that affect the rate and extent of: (a) phosphorylation of $E_r$ by phosphatase-1 kinase; (b) the autophosphorylation of E-P; and (c) the deactivation of $E_r$ are of potential importance in regulating the extent as well as duration of phosphatase-1 activity. Recently, DePaoli-Roach (18) reported that the R-subunit of phosphatase-1 could be phosphorylated at Ser residue by casein kinase II. The action of casein kinase II alone did not result in activation of the phosphatase. However, it could potentiate the extent of phosphorylation at Thr residue and activation of the phosphatase activity catalyzed by suboptimal concentrations of $F_A$.

The possibility that the requirement of Mg$^{2+}$ for autophosphorylation-activation reaction is particular to the thio phosphorylated enzyme intermediate is unlikely since removal of Mg$^{2+}$ by EDTA results in stabilization of both phosphorylated and thio phosphorylated enzyme intermediates (Fig. 1). Table I indicates that the ratio between phosphatase activity generated and $^{35}$S released from $[^{35}S]E-P$ with Mg$^{2+}$ as an activator is higher than that with Mn$^{2+}$. The data are interpreted to mean that Mg$^{2+}$, but not Mn$^{2+}$, is the natural cofactor for phosphatase-1. Therefore, Mg$^{2+}$ is efficiently incorporated into the active site at the correct position where it exerts the cofactor function more effectively than Mn$^{2+}$.

The interpretation is supported by the observations that Mn$^{2+}$ is only about 50-60% as effective as Mg$^{2+}$ in activation of phosphatase-1 holoenzyme catalyzed by $F_A$ with ATP as a substrate (6-8, 15, 16, 28, 26). The present results indicate that Mg$^{2+}$ is less effective than Mn$^{2+}$ in the activation of trypsinized phosphatase-1. The data may be explained by assuming that Mg$^{2+}$ but not Mn$^{2+}$ requires the action of R-subunit for its incorporation into the active site in an efficient and correct way. The possibility that Mn$^{2+}$ might exert its activating effect by a mechanism different from that of Mg$^{2+}$, however, cannot be excluded. All the current published data indicate that Mg$^{2+}$ alone is ineffective in activating phosphatase-1, either in the form of the free C-subunit or holoenzyme (1-3, 10, 15, 16, 23). The discrepancy may be due to differences in experimental conditions.

It has been suggested that the activation of phosphatase-1 by Mn$^{2+}$ may have resulted from a Mn$^{2+}$-catalyzed covalent modification of the enzyme, perhaps involving sulphydryl groups of the C-subunit (16). Such a reaction has been proposed by Yan and Graves (43). The present studies, however, favor the hypothesis that activation is accompanied by insertion of Mg$^{2+}$ into the C-subunits. Evidence that supports the hypothesis includes: (a) Mg$^{2+}$ is required for autophosphorylation-activation of E-P (Fig. 5), (b) deactivation of $E_r$ can be accelerated by a metal ion chelator, PP$_4$, whose effect can be blocked by Mg$^{2+}$ (Fig. 8), and (c) the free C-subunit produced by limited trypsinization can be activated by Mg$^{2+}$ (Fig. 9). It has been suggested that most of the enzymes involved in transferring phosphate groups should have at least one cationic group suitably disposed near the active site in order to facilitate the complexation and orientation of the phosphate group (36, 44). It therefore seems reasonable to assume that Mg$^{2+}$ may serve as a cationic site. Recently, we have shown that Ca$^{2+}$ and calmodulin act as allosteric activators in transforming phosphatase-2 (calcineurin) into a relaxed conformation which required the presence of Mg$^{2+}$ or a transition metal ion for expressing its catalytic activity (44-46) supporting the notion that Ca$^{2+}$ may represent an essential cofactor in catalysis for phosphoprotein phosphatases in general.

The regulation of phosphatase-1 by intracyclic activation-deactivation reaction via transient phosphorylation of the R-subunit represents a novel regulatory mechanism in covalent modification of enzymatic activity. In conventional covalent modification, phosphorylation reactions in locking the modified enzyme in either an active (e.g. phosphorylase a) or an inactive (e.g. glycogen synthase b) state and the reversal requires a separate phosphatase. By contrast, the intracyclic activation-deactivation mechanism involves the formation of a transient-activated form which requires continued utilization of ATP for enzyme phosphorylation to maintain the activated form. In such a mechanism, the activity of only the kinase in conjunction with the intrinsic activation-deactivation reactions determine the level and duration of the enzymatic activity. Such a mechanism, which involves a built-in automatic turn-off device, would be more sensitive than the conventional extracyclic phosphorylation-dephosphorylation
mechanism in controlling the duration of the activated state of the target enzyme. It is reasonable to speculate that the intracyclic phosphorylation-dephosphorylation mechanism is not unique to phosphatase-1 but may exist in regulation of many other enzymes. In addition, this type of regulation might operate in conjunction with a regulatory step which involves the conventional extracyclic phosphorylation-dephosphorylation mechanism.

Acknowledgments—We wish to thank Wanda W. S. Chan and Adam M. Brook for their excellent technical assistance.

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