Calmodulin-dependent protein phosphatase isolated from bovine brain consists of a catalytic subunit A (Mr = 60,000) and a regulatory subunit B (Mr = 19,000) present in equal molar ratios. The two subunits were dissociated by gel filtration in 6 M urea and reconstituted to investigate the role of calmodulin and subunit B in regulating the phosphatase activity of subunit A.

The activity of subunit A was stimulated 2-fold by calmodulin, 13-fold by subunit B, and 21-fold by both, indicating that the effects of both were synergistic. Maximum stimulation by calmodulin was observed at a calmodulin to subunit A molar ratio of 2:1 in the presence or absence of subunit B, whereas that by subunit B was observed at a B to A molar ratio of 3:1 in the presence or absence of calmodulin. Calmodulin and subunit B increased the V_max of subunit A 2- and 5-fold, respectively, but had little effect on the K_m for casein. The specific activity of the phosphatase reconstituted from subunits A and B reached 86% that of the native enzyme, whereas that of the holoenzyme reached 90%. Subunit B, even though similar to calmodulin in many respects, did not stimulate the activity of native phosphatase, suggesting that it cannot substitute for calmodulin. Limited trypsinization of subunit A increased its catalytic activity to the level observed with calmodulin; and this activity was further stimulated by subunit B but not by calmodulin. These results indicate that subunit A of phosphatase contains one catalytic domain and two distinct regulatory domains, one for calmodulin, and another for subunit B, that these two proteins do not substitute for one another and that they stimulate subunit A synergistically.

Protein phosphorylation and dephosphorylation play an important role in neuronal function (Ingebritsen and Cohen, 1983; Nestler et al., 1984). Calmodulin mediates many of the functions of Ca^{2+} (Cheung, 1980; Means et al., 1982; Manalan and Klee, 1984), including the regulation of protein phosphorylation, catalyzed by a calmodulin-dependent protein kinase (Nestler and Greengard, 1983), and of protein dephosphorylation, catalyzed by a calmodulin-dependent phosphatase (Stewart et al., 1982; Yang et al., 1982). The phosphatase, also known as modulator-binding protein (Wang and Desai, 1977; Sharma et al., 1979), CaM'-BP_50 (Wallace et al., 1980), and calcineurin (Klee et al., 1979), consists of subunits A (Mr = 60,000) and B (Mr = 19,000) present in equal molar ratio (Wallace et al., 1979). Subunit A binds calmodulin (Wang and Desai, 1977; Klee et al., 1979; Sharma et al., 1979) and contains the catalytic site (Winkler et al., 1984). Subunit B binds 4 Ca^{2+} ions with K_d = 10^{-6} M (Klee et al., 1979), has an NH_2 terminus blocked by a myristyl group (Aitken et al., 1982), and possesses 35% sequence homology with calmodulin (Aitken et al., 1984). The enzyme appears to have broad substrate specificity, dephosphorylating a wide variety of proteins (Stewart et al., 1982; Yang et al., 1982; Klee et al., 1983; Chernoff et al., 1984; King et al., 1984), including p-nitrophosphoryl phenylphosphate and phosphotyrosine (Pallen and Wang, 1983). Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Mg^{2+} have been reported to stimulate the enzyme (King and Huang, 1983; Klee et al., 1983; Pallen and Wang, 1983, 1984; Tallant and Cheung, 1984a, 1984b; Gupta et al., 1984; Merat et al., 1984; Li, 1984; and Chan, 1984). Although native phosphatase is stimulated by Ca^{2+} and calmodulin, subunit A isolated by gel filtration in 6 M urea and 15 mM EDTA shows no activity in the presence of Ca^{2+} unless Mn^{2+} is included in the assay (Winkler et al., 1984). These findings, together with those of King and Huang (1984) who noted that the phosphatase contained stoichiometric amounts of zinc and iron, suggest that the phosphatase may be a metalloenzyme.

Calmodulin-dependent protein phosphatase has been detected in many mammalian tissues, and its activity appears to be highest in the brain, especially the neostriatum (Wallace et al., 1980). On a subcellular basis, the enzyme is localized at post-synaptic densities and microtubules of post-synaptic dendrites (Wood et al., 1980). Ontogenetic studies on rat brain and chick brain and retina indicate that its synthesis correlates with the period of major synaptogenesis (Tallant and Cheung, 1983). Collectively, these results suggest that the enzyme has an important role in certain synaptic functions.

We have previously resolved calmodulin-dependent phosphatase into its catalytic subunit A and regulatory subunit B (Winkler et al., 1984). The resolution of the two subunits affords us an opportunity to study further the regulatory properties of the enzyme. In this paper, we report on the reconstitution of the holoenzyme from its subunits and the

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\( \text{CaM}, \text{calmodulin} \); BSA, fatty acid-free bovine serum albumin; EGTA, ethyleneglycol bis(beta-amino-ethly ether)-N,N',N''-tetraacetic acid; SDS, sodium dodecyl sulfate; Mes, 2-(N-morpholino)ethanesulfonic acid.
effect of calmodulin, subunit B, or both on the phosphatase activity of subunit A.

Experimental Procedures

Materials—Calmodulin-dependent protein phosphatase was purified to homogeneity from bovine brain (Tallant et al., 1983). The catalytic subunit of CAMP-dependent protein kinase, isolated from bovine heart (Reimann and Beham, 1983), was used to catalyze the phosphorylation of casein to a specific activity of 0.04 pCi/nmol. Bovine heart calmodulin was purified to homogeneity using a phenyl-Sepharose column (Gopalakrishna and Anderson, 1982). [γ-32P]ATP (specific activity 2500-2900 Ci/mmol ATP) and methyl-14C-methylated bovine serum albumin were from New England Nuclear; molecular weight markers and Sephacryl S-200 from Pharmacia; fatty acid-Tris-ATP, turkey egg white type II trypsin inhibitor, and yeast dithiothreitol, was dialyzed against 0.5 liter of buffer A (50 mM Tris-HCl (pH 7.0), 0.5 mM dithiothreitol, was dialyzed against one liter of buffer A containing 0.5 M urea, 0.5 mM dithiothreitol, 1.5 mM CaCl2, 0.7 mM EDTA, 6 M urea, 100 mM LiBr, and 0.02% NaN3) for 7 h with several changes. The dialyzed phosphatase was loaded onto a Sephacryl S-200 column (1.0 × 117 cm) previously equilibrated with buffer B. Fractions of 1.1 ml were collected at a flow rate of 4-5 ml/h. Two protein peaks were obtained. The first peak fractions contained only subunit A, and these fractions were dialyzed as follows for storage at -20 °C: 3 volumes of the eluent were mixed with 6.8 volumes of buffer A and 0.2 volume of 5 mg/ml BSA.

The second peak fractions, containing subunit B and usually a trace of subunit A, were pooled (4 ml) and dialyzed sequentially against one liter of buffer A containing 0.5 M urea for 2 h, one liter of buffer A containing 60% glycerol for 2 h (to concentrate the sample), 0.5 liter of buffer A containing 0.5 M urea for 1 h, and 0.5 liter of buffer B for 7 h with several changes. The dialyzed phosphatase was loaded onto a Sephacryl S-200 column (1.0 × 117 cm) previously equilibrated with buffer B. Fractions of 1.1 ml were collected at a flow rate of 4-5 ml/h. The fractions containing only subunit B were pooled (3.3 ml) and dialyzed sequentially against 0.5 liter of buffer A containing 0.5 M urea for 1.5 h; 0.5 liter of buffer A containing 60% glycerol for 1-2 h; and 1 liter of buffer A containing 0.2 M urea for 7 h with 3 changes. The dialyzed sample was stored at -20 °C. All other procedures were done at 4 °C.

Assay of Protein Phosphatase Activity—Phosphatase activity was measured at 30 °C essentially as before (Tallant and Cheung, 1984a); the detailed conditions are provided in the figure legends. Assays were run from 5 to 10 min, depending on the activity of each preparation, to ensure that the amount of substrate dephosphorylated was less than 15%. The final concentrations of components in the phosphatase assay mixture were: Tris-HCl (pH 7.0), 50 mM; dithiothreitol, 0.5 mM; CaCl2, 0.6 mM; MnCl2, 0.7 mM; EDTA, 0.3 mM; urea, 150 mM; BSA, 1 mg/ml; and [γ-32P]casein, 15 µM. Additional components are given in the figure and table legends. Specific activity is defined as nanomoles of 32P released per mg of protein per min.

Stimulation of Subunit A and Reconstituted A-B by Calmodulin—Subunit A isolated from the Sephacryl S-200 column in 6 M urea showed no phosphatase activity in the presence of Ca2+, or calmodulin, or both, unless Mn2+ was added. The protein sample which had been diluted to 150 µl with H2O. After a 60-min incubation at 0 °C, the protein was pelleted and the supernatant discarded. The pellet was dissolved in 24 µl of a buffer containing 8.3 mM Tris-HCl, 0.33 mM NaOH, 0.0025% bromphenol blue, 1.25 mM EGTA, 12.5% β-mercaptoethanol, 1.25% SDS, and 1.5% sucrose. Electrophoresis was performed in 0.1% SDS and 1 mM EGTA according to Laemmli (1970) using a 7.5-15% acrylamide gradient slab gel containing 0.8% bisacrylamide. The gel was stained with Coomassie Brilliant Blue R-250.

Results

Stability and Purity of Subunits A and B—The isolated subunit A was unstable, and its phosphatase activity was completely lost after several weeks at -20 °C. However, supplementing the sample with BSA (0.1 mg/ml) and 1.8 M urea preserved the enzyme activity for several months at -20 °C. Occasionally, subunit A was inactive or lost activity within days during storage at -20 °C. The reason for the rapid loss of enzyme activity is not clear.

Subunit B obtained from the Sephacryl S-200 column was invariably contaminated by a trace of subunit A. Rechromatography on a second Sephacryl S-200 column rendered subunit B completely free of subunit A. Subunit B was stable for several months when stored at -20 °C in buffer A containing 0.2 M urea; however, repeated freezing and thawing resulted in a gradual loss of its ability to stimulate subunit A. Subunit B is blocked at its amino terminus by myristic acid, and appears to adhere to surfaces (Winkler et al., 1984).

The gel patterns of subunits A and B are shown in Fig. 1; each appears to be free of the other.

FIG. 1. Electrophoretic profile of subunits A and B in SDS-polyacrylamide gel. Lane 1, molecular weight markers: from the top, phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,000); Lane 2, native phosphatase, 10 µg of protein; Lane 3, subunit A, 10 µg of protein; Lane 4, subunit B, 1-2 µg of protein. (The amount of protein applied in Lane 4 is approximate; subunit B might not have been quantitatively pelleted from a dilute solution. See "Experimental Procedures"). The molecular weight of subunit B in SDS gel corresponds to 16,500, somewhat lower than the 19,000 determined from amino acid analysis (Klee et al., 1982).
Regulation of Calmodulin-dependent Phosphatase

present (Winkler et al., 1984; Merat et al., 1984). In all subsequent experiments, the assay mixture contained 0.7 mM Mn²⁺.

Fig. 2 shows the time-dependent stimulation of subunit A by calmodulin. Subunit A was preincubated with calmodulin for various times before the substrate was added to initiate the phosphatase assay. Phosphatase activity reached a maximum after about 20 min of preincubation, and remained elevated thereafter. In the control without calmodulin, subunit A activity remained unchanged throughout the course of the preincubation. The length of preincubation required for maximum stimulation by calmodulin of subunit A varied with phosphatase preparation from 20 min to as long as 3 h, possibly because of a difference in its state of renaturation from the harsh conditions of isolation. As a comparison, native phosphatase was preincubated with calmodulin under comparable conditions, and the stimulation appeared to be virtually instantaneous, an observation similar to that of other calmodulin-dependent enzymes such as phosphodiesterase (Cheung, 1971), adenylate cyclase (Lynch et al., 1976), and Ca²⁺-ATPase (Lynch and Cheung, 1979).

The stimulation of subunit A as a function of calmodulin concentration was examined in Fig. 3. Calmodulin increased subunit A activity from 0.28 to 0.78 nmol/mg/min at a calmodulin to subunit A molar ratio of 2:1, and the activity remained constant thereafter. When subunit B was present during preincubation (i.e. to generate the A-B complex), the phosphatase activity increased severalfold, and calmodulin further enhanced the activity, reaching a maximum also at a calmodulin to subunit A ratio of 2:1. At higher ratios, phosphatase activity was slightly inhibited, but this activity was still markedly higher than the activity obtained in the absence of subunit B. The slight inhibition may result from interference of the binding of B to subunit A by calmodulin, but this possibility appears unlikely. The same ratio of calmodulin to subunit A gave maximal stimulation of subunit A in the absence or presence of subunit B (also see next section). These results imply that subunit A has two distinct regulatory domains, one for calmodulin and another for subunit B, and that the effects of both domains appear to be synergistic.

**Stimulation of Subunit A Activity by Subunit B—Fig. 4**

![Fig. 2. Time-dependent stimulation of subunit A by calmodulin. Subunit A (140 nM) was preincubated with or without calmodulin (1.12 μM) at 30 °C in 20 μl of 50 mM Tris-HCl (pH 7.0) containing 0.5 mM dithiothreitol, 1.5 mM CaCl₂, 0.7 mM EDTA, 0.1 mM urea, and 2.5 mg/ml BSA for the times indicated. As a comparison, native phosphatase (31.2 nM) was preincubated with 400 nM calmodulin similarly. Phosphatase activity was initiated by the addition of 30 μl of 50 mM Tris-HCl (pH 7.0) containing 0.5 mM dithiothreitol, 1.16 mM MnCl₂, 0.1 mg/ml BSA, and 25 μM [³²P]casein. The incubation was terminated after an additional 10 min.](image)

![Fig. 3. Stimulation of subunit A and reconstituted A.B by calmodulin. Subunit A (140 nM) was preincubated with or without subunit B (280 nM) in 20 μl of 50 mM Tris-HCl (pH 7.0) containing 0.5 mM dithiothreitol, 1.5 mM CaCl₂, 0.7 mM EDTA, 0.1 mM urea, and 2.5 mg/ml BSA at 30 °C for 60 min. Next, 20 μl of 50 mM Tris-HCl (pH 7.0) containing 0.8 mM dithiothreitol, 1.7 mM MnCl₂, 0.1 mg/ml BSA, and various concentrations of calmodulin was added. After 20 min, 10 μl of 75 μM [³²P]casein in 50 mM Tris-HCl (pH 7.0) was added to initiate the reaction. The reaction was terminated after an additional 10 min. Both subunits were isolated from the same phosphatase preparation.](image)

![Fig. 4. Time-dependent stimulation of subunit A by subunit B. Subunit A (98 nM) was preincubated with or without subunit B (295 nM) at 30 °C in 20 μl of 50 mM Tris-HCl (pH 7.0) containing 0.5 mM dithiothreitol, 1.5 mM CaCl₂, 0.7 mM EDTA, 0.1 mM urea, and 2.5 mg/ml BSA for the times indicated. Phosphatase activity was initiated by the addition of 30 μl of 50 mM Tris-HCl (pH 7.0) containing 0.5 mM dithiothreitol, 1.16 mM MnCl₂, 0.1 mg/ml BSA, and 25 μM [³²P]casein. The incubation was terminated after an additional 5 min.](image)

shows the time-dependent stimulation of subunit A by subunit B. Phosphatase activity increased from a specific activity of 1 to a maximum of 15 in 5 h, and remained unchanged thereafter, reminiscent of the results obtained with calmodulin in Fig. 2. In experiments using other phosphatase preparations, the length of preincubation needed for maximum stimulation varied from 1 to 4 h. In general, the time of preincubation for subunit A with subunit B giving maximum stimulation was considerably longer than that for subunit A with calmodulin (see Fig. 2). In the experiment described in Fig. 4, the phosphatase activity of subunit A increased slightly with time, possibly due to a slow renaturation. This increase was not necessarily observed with other preparations (see for example Fig. 2).

Fig. 5 examines the effect of subunit B concentration on subunit A activity. The phosphatase activity increased with increasing concentrations of subunit B during preincubation, reaching a maximal level at a B:A molar ratio of 3:1. At ratios exceeding 4:1, subunit A activity decreased. When calmodulin was added after preincubation, subunit A activity was further
The activities were calculated on the basis of the protein concentration of subunit A. B. CaM) are included for comparison. The activities relative to preparation, as did the effectiveness of calmodulin and the enzyme used in this experiment.

It was 450 nM, and that of native phosphatase was 15 nM. In tubes containing no subunit A, the final concentration of subunit B was 200 nM and that of calmodulin was 750 nM. The specific activities were calculated on the basis of the protein concentration of subunit A. The specific activities of native phosphatase (A.B) and the holo-enzyme (A-B-CaM) are included for comparison. The activities relative to subunit A alone (assigned an arbitrary value of 1) are given in parentheses. The subunits were isolated from the native phosphatase used in this experiment.

TABLE I
Stimulation of subunit A activity by calmodulin or by subunit B

| Additions          | Phosphatase activity (nmol/mg/min) |
|--------------------|-----------------------------------|
|                    |                                   |
| B                  | 0                                 |
| B + CaM            | 0                                 |
| A                  | 0.3 (1)                           |
| A + CaM            | 0.9 (3)                           |
| A + B              | 3.7 (12)                          |
| A + B + CaM        | 9.0 (30)                          |
| A-B (native phosphatase) | 4.3 (14)                       |
| A-B-CaM (native holoenzyme) | 22.4 (75)                    |

increased, but the maximal activity level was also achieved at a B:A ratio of 3:1, and the pattern was qualitatively similar. In other experiments using a different preparation of subunit A, maximal stimulation was obtained at a B:A ratio of 2:1. Since subunit A activity decreased in the absence or presence of calmodulin, the inhibition does not appear to result from interference of binding of B to A by calmodulin. The mechanism for the apparent inhibition remains to be investigated further.

The stimulation of subunit A by calmodulin or by subunit B is compared in Table I. Subunit A has a low activity, and subunit B has no detectable activity, even in the presence of calmodulin. Both calmodulin and subunit B stimulated the activity of subunit A, but the stimulation by subunit B was much more pronounced (see also Figs. 3 and 5). In the presence of both, subunit A displayed the highest activity, clearly indicating that their effects were synergistic.

The specific activity of subunit A varied from preparation to preparation, as did the effectiveness of calmodulin and subunit B to stimulate subunit A. However, the relative effects of calmodulin and subunit B on subunit A were always reproducible.

Although we have taken precautions to purify phosphatase from bovine brain to apparent homogeneity, sometimes a preparation might have been contaminated with a trace of tissue protease. Such a preparation upon storage would reveal faint extra protein bands in an SDS gel, and the subunits isolated from them would not support good reconstituted phosphatase activity, and were not used in our reconstitution experiments.

Distinct Calmodulin-binding and Subunit B-binding Sites of Subunit A—The above experiments suggest that calmodulin and subunit B did not substitute for each other in stimulating the activity of subunit A. It is conceivable that even if the calmodulin binding site of subunit A in native phosphatase recognizes subunit B, the isolated subunit A may fail to do so. Subunit A was prepared under harsh conditions and might not have completely renatured to its original native conformation in its isolated subunit form.

To examine this possibility, the effect of subunit B on the activity of native phosphatase was studied (Fig. 6). Subunit B did not affect the basal phosphatase activity, even at a high molar ratio of 24:1. When calmodulin was present in the assay mixture, phosphatase activity increased severalfold, but subunit B did not affect the stimulated phosphatase activity. These results indicate that the calmodulin binding site of native phosphatase did not recognize subunit B, nor did subunit B interfere with the stimulation of native phosphatase by calmodulin. We thus conclude that the calmodulin binding site of subunit A is distinct from its subunit B binding site.

Another approach to distinguishing the two regulatory sites is to take advantage of the stimulatory effect of controlled trypsinization on phosphatase activity. This renders phosphatase insensitive to calmodulin (Manalan and Klee, 1983; Tallant and Cheung, 1984b), presumably because the calmodulin-binding site was preferentially destroyed. Controlled trypsinization of subunit A also stimulated its activity and rendered it insensitive to calmodulin (Merat et al., 1984). In the experiment presented in Table II, calmodulin slightly increased the phosphatase activity of trypsined subunit A, indicating that subunit A was under-trypsinized; in other

![FIG. 5. Stimulation of subunit A by subunit B in the presence or absence of calmodulin. Subunit A (96 nM) was preincubated in 20 μl of 50 mM Tris-HCl (pH 7.0) containing 0.5 mM dithiothreitol, 1.5 mM CaCl2, 0.7 mM EDTA, 370 mM urea, 2.5 mg/ml BSA, and various concentrations of subunit B at 30 °C for 4.5 h. Next, 20 μl of 50 mM Tris-HCl (pH 7.0) containing 0.8 mM dithiothreitol, 1.7 mM MnCl2, and 0.1 mg/ml BSA with or without 400 nM calmodulin was added. After 5 min, 10 μl of 75 μM [32P]casein in 50 mM Tris-HCl (pH 7.0) was added to initiate the reaction. The reaction was terminated after another 5 min.](image)

![FIG. 6. Effects of calmodulin and subunit B on the activity of native phosphatase. Native phosphatase (31.2 nM) was preincubated in 20 μl of 50 mM Tris-HCl (pH 7.0) containing 0.5 mM dithiothreitol, 1.5 mM CaCl2, 0.7 mM EDTA, 370 mM urea, 2.5 mg/ml BSA, and various concentrations of subunit B at 30 °C for 60 min. Next 20 μl of 50 mM Tris-HCl (pH 7.0) containing 0.8 mM dithiothreitol, 1.7 mM MnCl2, and 0.1 mg/ml BSA with or without 400 nM calmodulin was added. (Optimal activation of native phosphatase under these conditions was observed at a calmodulin to enzyme ratio of 13:1.) After 5 min, 10 μl of 75 μM [32P]casein in 50 mM Tris-HCl (pH 7.0) was added to initiate the reaction. The reaction was terminated after another 5 min.](image)
Experimentally, in which the time of trypsinization was better controlled, no calmodulin effect was observed. Such a trypsin-stimulated subunit A was still clearly responsive to subunit B. Compared to the untrypsinized subunit A, however, the effect of subunit B on the trypsinized subunit A was smaller, suggesting that trypsinization might have slightly damaged the binding site of subunit A for subunit B.

Collectively, these results indicate that the calmodulin and the subunit B binding domains of subunit A are distinct, and that calmodulin does not compete with B for its binding site; neither does B compete with calmodulin for its binding site.

Effect of Calmodulin and Subunit B on Kinetic Parameters of Subunit A—The effects of calmodulin and subunit B on the kinetic parameters of subunit A were examined with the Lineweaver-Burk plot (Fig. 7). The maximal velocity ($V_{max}$) for the catalytic activity of subunit A assayed alone was 4.1 nmol/mg/min. In the presence of calmodulin, the $V_{max}$ increased to 6.9, and in the presence of subunit B, to 18.0. The apparent $K_m$ of subunit A for casein was 5.2 μM, and was unchanged in the presence of calmodulin. In the presence of subunit B, the apparent $K_m$ decreased slightly to 4.2 μM. In general, the effect of both proteins on subunit A appears to be primarily on the $V_{max}$ rather than the $K_m$. The effect of calmodulin on the kinetic parameters of native phosphatase (Tallant and Cheung, 1984a) appears similar.

Efficiency of Reconstitution of Subunits A and B—The stimulation of subunit A by subunit B suggests that subunits A and B can be reconstituted to form the A-B complex. Similarly, the stimulation of subunit A by subunit B and by calmodulin suggests reconstitution of the holoenzyme (A-B-CaM).

One way to demonstrate the formation of the A-B complex is to determine the apparent molecular weight of the reconstituted enzyme on a sucrose density gradient. A mixture of subunits A and B at a molar ratio of 1:2 was preincubated for 1 h and then centrifuged on a 5-20% sucrose density gradient. With methyl-3H-C-methylated BSA as an internal marker, the reconstituted enzyme gave a molecular weight of 73,000. In a parallel experiment, the native phosphatase gave a molecular weight of 78,000 (see also Wallace et al., 1978, 1979; Tallant and Cheung, 1984a). The apparently lower molecular weight suggests that the reconstituted enzyme assumes a conformation slightly different from that of the native enzyme, probably due to an incomplete renaturation of the two subunits.

Although reconstituted A-B appears to have a slightly smaller molecular weight than that of native phosphatase, it is stimulated by calmodulin in a manner qualitatively similar to that of native phosphatase (see, for example, Table I). Moreover, the response of the reconstituted A-B to trypsinization also appears comparable. The activity of reconstituted A-B was increased by controlled trypsinization from 11.5 to 20.4, a value comparable to that (18.9) obtained in the presence of calmodulin (see Table II), indicating that trypsinization-stimulated the reconstituted A-B and rendered it insensitive to calmodulin, as it did native phosphatase (Tallant and Cheung, 1984b).

To estimate the efficiency of reconstitution, we compared the specific activity of the enzyme reconstituted from subunits A and B with that of the native enzyme on the basis of the concentration of subunit A. Table I shows that the specific activities of the native phosphatase in the absence and presence of calmodulin were 4.3 and 22.4, respectively, whereas those of the reconstituted enzyme were 3.7 and 9.0. These values suggest that the efficiency of reconstitution for the basal activity was 86% and that for the calmodulin-stimulated activity was 40%. Thus, reconstitution of the basal activity (A-B) appeared more efficient than that of the holoenzyme (A-B-CaM). The harsh conditions required for dissociation of the two subunits might have denatured one or both of the subunits, and reconstitution to form the A-B complex might have been easier to achieve than the A-B-CaM complex.

In fact, the conditions of reconstitution affected the efficiency of reconstitution and thus the final phosphatase activity. In Table III, we have examined the effect of the order of addition of components to the reconstitution mixture on the phosphatase activity. For example, when A and calmodulin were present in the reconstitution mixture, and B was added later to the assay mixture, the phosphatase activity was 9.2 nmol/mg/min, whereas when A and B were present in the reconstitution mixture, and calmodulin was later added to the reconstitution mixture.
assay mixture, the phosphatase activity was 21.4. The higher activity in the latter case was possibly due to the faster stimulation of subunit A by calmodulin (compare Fig. 2 with Fig. 4). When A, B, and calmodulin were present simultaneously in the reconstitution mixture, phosphatase activity increased to 36.5. These results clearly show that the simultaneous presence of A, B, and CaM affords more efficient reconstitution. Since the native phosphatase from which the two subunits were isolated had a specific activity of 40.7 in the presence of calmodulin, the efficiency of reconstitution of the holoenzyme corresponded to 90% under these conditions. The efficiency of reconstitution for the basal activity of this preparation was 74%. These values should be taken as representative; the efficiency of reconstitution varied considerably from preparation to preparation.

**DISCUSSION**

In this study, we have resolved the catalytic subunit A of calmodulin-dependent protein phosphatase from its regulatory subunit B, using rather harsh conditions, and have defined certain parameters under which they retain their biological activities. These conditions are probably not optimal; occasionally the isolated subunits were relatively inactive or lost activity rapidly after isolation. Nevertheless, we have successfully determined, albeit preliminarily, the qualitative effect of calmodulin and subunit B on the phosphatase activity of subunit A. Our experiments demonstrate that subunit A carries two distinct regulatory domains, one for calmodulin, and another for subunit B, with neither substituting for the other. Occupation of each of these sites stimulates subunit A to a certain level, and the effect of subunit B appears larger than that of calmodulin. Maximal stimulation, however, requires the occupancy of both sites, but the sequence of occupancy by calmodulin or by subunit B does not appear to affect

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**TABLE III**

| Additions to reconstitution mixture | Additions to assay mixture | Phosphatase activity nmol/mg/min |
|------------------------------------|---------------------------|--------------------------------|
| A                                  | CaM                       | 3.7 (1)                        |
| A + CaM                            | B                         | 3.7 (2)                        |
| A + B                              | B + CaM                   | 3.9 (2)                        |
| A + CaM                            | B                         | 9.2 (5)                        |
| A + B + CaM                        | CaM                       | 21.4 (13)                      |
| Native phosphatase                 | CaM                       | 18.7 (11)                      |

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FIG. 8. Scheme showing various activated states of phosphatase. The activity of subunit A is assigned a value of 1; those of the other states of subunit A are expressed relative to this value. The specific activity of subunit A was 0.9; those of the native phosphatase from which the subunits A and B were isolated were 19 and 41 in the absence or presence of calmodulin, respectively. A, subunit A; A’, trypsinized subunit A; B, subunit B; A-B, reconstituted phosphatase; (A-B)’, trypsinized reconstituted phosphatase; A’-B, trypsinized A reconstituted with B; CaM, calmodulin. The time course of reconstitution of the experiments described here was different from that in Table III; the data are thus not directly comparable. Since the time course of reconstitution of A-CaM with subunit B appears to be slower than that of A-B with calmodulin (see Table III), longer incubations were required to achieve comparable final activities.

The final achievable activity. The effects of calmodulin and subunit B appear to be synergistic. As noted previously (Winkler et al., 1984), subunit A as isolated displayed no phosphatase activity unless Mn^2+ was present. The holoenzyme (A-B-CaM) could be reconstituted by presenting subunit A first with calmodulin and then with subunit B, or first with subunit B and then with calmodulin, although as indicated in Table III, the reconstituted activity appeared higher when calmodulin was added to the A-B complex than when subunit B was added to the A-CaM complex. However, the holoenzyme (A-B-CaM) formed either by way of the intermediate complex A-CaM or A-B could display a comparable activity level, provided that sufficient time was allowed to achieve maximum stimulation (Fig. 8). This suggests that the sequence of occupation of the two regulatory sites of subunit A did not affect the final achievable activity, even though the activity of the intermediate complex A-B was considerably higher than that of A-CaM. In contrast, trypsination of the reconstituted A-B (A-B’) gave an activity substantially higher than trypsinized A reconstituted with B (A’-B). Trypsinization of subunit A alone may have partially damaged the catalytic site, or the subunit B binding site, or both, thus impairing its catalytic activity, or its ability to reconstitute with subunit B. Conceivably, the presence of subunit B in the A-B complex might have partially protected these sites from any untoward effects of trypsination.

Kinetic experiments show that both calmodulin and subunit B increase the $V_{max}$ of subunit A, with little or no effect on its apparent $K_m$ for casein. These findings are qualitatively similar to the effect of calmodulin on native phosphatase (Tallant and Cheung, 1984a). The apparent $K_m$ of phosphatase for calmodulin is 30 nM (Tallant and Cheung, 1984a), and appears comparable to that of subunit A for calmodulin. Indeed the finding (Fig. 3) that approximately the same concentration of calmodulin in the absence or presence of subunit B gave half-maximal stimulation of subunit A sup-

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2 A more detailed description of the effect of the conditions of reconstitution on phosphatase activity will be described elsewhere (D. L. Merat and W. Y. Cheung, manuscript in preparation).

3 D. L. Merat and W. Y. Cheung, unpublished experiment.
ports the notion that subunit B did not affect the affinity of subunit A for calmodulin. Likewise, the experiment in Fig. 5 suggests that calmodulin did not materially affect the affinity of subunit A for subunit B. Collectively, these findings suggest that the two regulatory sites of subunit A are not only distinct but also independent of each other.

The subunit structure of calmodulin-dependent phosphatase is unique among calmodulin-dependent enzymes. The catalytic activity of subunit A depends not only on its intrinsic regulatory subunit B, but also on calmodulin. Subunit B is in many respects similar to calmodulin, including its physicochemical properties and sequence homology, but appears to be biologically distinct from calmodulin. Subunit B does not stimulate native phosphatase.

Subunit A in native phosphatase binds subunit B tenaciously; high concentrations of urea are required to resolve them. In contrast, the binding of calmodulin to native phosphatase appears readily reversible, apparently dependent on the concentration of Ca$^{2+}$.

The phosphatase in situ would appear to exist as an A-B complex which has relatively low enzyme activity. Complex formation with calmodulin in response to an increase in intracellular Ca$^{2+}$ elevates its enzyme activity markedly. According to this view, which is believed to apply to nearly all other calmodulin-dependent enzymes (Cheung, 1980; Klee and Vanaman, 1982), the rate-limiting factor appears to be the intracellular level of Ca$^{2+}$.

Phosphatase is sensitive to other divalent cations, and the effect of various divalent cations on phosphatase activity appears to be complex, having received considerable attention from many groups of investigators (King and Huang, 1983, 1984; Klee et al., 1983; Pallen and Wang, 1983, 1984; Tallant and Cheung, 1984a, 1984b; Gupta et al., 1984; Merat et al., 1984; Li, 1984; Li and Chan, 1984). They have noted that Mn$^{2+}$, Ni$^{2+}$, and Mg$^{2+}$ stimulated phosphatase activity and that the enzyme (A-B) contains stoichiometric amounts of Zn and Fe. It is not known whether the two cations reside in subunit A or subunit B, or both. We have found that subunit A requires Mn$^{2+}$ for activity and that Ni$^{2+}$ and Co$^{2+}$ do not substitute well for Mn$^{2+}$ (Winkler et al., 1984; Merat et al., 1984). It remains to be shown if subunit B, which is reported to bind Ca$^{2+}$ (Klee et al., 1979), plays a role in determining the cation requirement of phosphatase.

Although the present investigation shows the separate stimulatory effects of calmodulin and subunit B on subunit A, the exact role of subunit B in regulating phosphatase activity is not apparent. One role may be to stabilize subunit A; another to amplify the effect of calmodulin, since the effect of calmodulin itself on subunit A appears to be rather minimal. A third may be that subunit B mediates the effect of certain metals, including that of Ca$^{2+}$. Further experiments are required before we can define clearly the role of calmodulin, subunit B, and divalent cations in the regulation of enzyme activity.

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