Concerted Regulation of Cardiac Sarcoplasmic Reticulum Calcium Transport by Cyclic Adenosine Monophosphate Dependent and Calcium–Calmodulin-Dependent Phosphorylations†

Christian J. Le Peuch, Jacques Haiech, and Jacques G. Demaille*

ABSTRACT: The rate of calcium uptake by canine cardiac sarcoplasmic reticulum is regulated by both a cyclic adenosine monophosphate (cAMP) dependent phosphorylation and a Ca^{2+}–calmodulin-dependent phosphorylation. The pure catalytic subunit of cAMP-dependent protein kinase catalyzes directly the incorporation of phosphate into phospholamban. This phosphorylation is not Ca^{2+}-dependent. In the presence of Ca^{2+} and calmodulin, a membrane-bound protein kinase catalyzes the incorporation of the same amount of phosphate into phospholamban at a different site. The latter covalent modification is totally dependent on Ca^{2+} and on exogenous calmodulin, with the half-maximal initial rate at 70 nM calmodulin. Calmodulin binding sites are shown to be present on sarcoplasmic reticulum vesicles by using [^{125}I]calmodulin. The membrane-bound calmodulin-dependent kinase is not inhibited by the cAMP-dependent protein kinase inhibitor. It does phosphorylate exogenous glycogen phosphorylase *b*. Conversely, exogenous glycogen phosphorylase *b* kinase catalyzes phospholamban phosphorylation beyond the activation provided by the attached δ subunit (calmodulin) alone. When fully phosphorylated, phospholamban is dephosphorylated by phosphoprotein phosphatase with biphasic kinetics. Whereas the Ca^{2+}–Mg^{2+}–ATPase activity is not modified by the covalent modifications of phospholamban, the rate of Ca^{2+} uptake is increased under conditions which induce phospholamban phosphorylation by the calmodulin-dependent kinase. This effect is amplified by the cAMP-dependent phosphorylation. A hypothetical model is presented for the functional role of these phosphorylations according to which each ATPase monomer interacts with one phospholamban monomer of M_r 11 000. The short stretch of phospholamban exposed at the outer surface of the vesicles contains both sites of phosphorylation, perhaps in a double-headed configuration. The membrane-bound kinase, similar but not identical to phosphorylase kinase, acts as a sensor of the cytosolic free Ca^{2+} concentration, whereas the cAMP-dependent direct phosphorylation provides an explanation for the acceleration of relaxation induced by catecholamines. There is no evidence for a metabolic cascade that would involve sequentially cAMP-dependent and calmodulin-dependent protein kinases.

The Ca^{2+}-dependent ATPase (EC 3.6.1.3, ATP phosphohydrolase) of canine cardiac sarcoplasmic reticulum is activated upon incubation with cAMP[1] and cAMP-dependent protein kinase (EC 2.7.1.37, ATP:protein phosphotransferase) (Wray et al., 1973; Kirchberger et al., 1974).

The stimulation of Ca^{2+}-dependent ATPase and calcium uptake is associated with phosphorylation of phospholamban, a membrane protein of M_r 22 000 (Kirchberger & Tada, 1976; Tada et al., 1979). Phospholamban can be phosphorylated by either exogenous or endogenous protein kinase (Wray & Gray, 1977; Schwartz et al., 1976; Will et al., 1976). Up to now, however, it has never been shown whether phospholamban is a substrate of the catalytic subunit of cAMP-dependent protein kinase or whether the C subunit activates another kinase that in turn phosphorylates phospholamban and is presumably Ca^{2+} dependent and similar to glycogen phosphorylase *b* kinase (Wray & Gray, 1977). Such a pathway would be reminiscent of the metabolic cascade that triggers glycogenolysis in response to catecholamines (Fischer et al., 1976). Most cardiac SR preparations used in the previous studies were cardiac microsomes, prepared according to Harigaya & Schwartz (1969). While developing methodology toward more extensive purification of cardiac SR, we noticed that, in purified SR vesicles, phospholamban is still rapidly phosphorylated by the pure catalytic subunit (C subunit) of cAMP-dependent protein kinase, but without significant activation of calcium uptake. Calmodulin, the ubiquitous Ca^{2+}-dependent regulator, has been recently identified as the δ subunit of glycogen phosphorylase *b* kinase (Cohen et al., 1978). It has also been shown to stimulate Ca^{2+} transport in cardiac SR vesicles (Katz & Remtulla, 1978). Experiments were therefore conducted to check whether phospholamban is also a substrate of a calmodulin-dependent protein kinase. The present report provides evidence that phospholamban may be phosphorylated not only by the catalytic subunit of cAMP-dependent protein kinase but also by a calmodulin-dependent membrane-bound protein kinase, presumably at a different site. The latter phosphorylation is of prime importance in the stimulation of calcium uptake.

Materials and Methods

Materials

Bovine serum albumin, cytochrome *c*, oligomycin, a histone II-A mixture, PMSF, and cAMP were from Sigma. Potato acid phosphatase was from Boehringer. Carrier-free [^{32}P]-orthophosphoric acid and [^{125}I]iodine (13–17 Ci/mg) were

† From the Centre de Recherches de Biochimie Macromoléculaire du CNRS, 34033-Montpellier, France. *Received June 20, 1979.* This work was supported in part by grants from CNRS (ATP modulation de l'action des hormones au niveau cellulaire), from INSERM (CRL 78-4-086-1 and 79.4.151.3, and ATP 63.78.95, "biologie et pharmacologie moléculaire et cellulaire de la fibre musculaire cardiaque normale et pathologique"), from DGRST (ACC Pharmacobiochimie des Polypeptides and ACC Biologie et Fonction du Myocarde), from NATO, and from Fondation pour la Recherche Médicale Francaise. C.J.L. is Attaché de Recherche INSERM.

[1] Abbreviations used: SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol, Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; C subunit, the catalytic subunit of cAMP-dependent protein kinase; cAMP, cyclic adenosine 3′,5′-monophosphate.

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purchased from NEN and Amersham, respectively. [γ-^{32}P]-ATP (~400 Ci/mol) was synthesized according to Glynn & Chappell (1964); $^{45}CaCl_2$ (>20 mCi/g) was obtained from CEA, Gif-sur-Yvette. Polyacrylamide gel electrophoresis reagents were from Bio-Rad. All other chemicals were from Merck or analytical grade.

The catalytic subunit of cAMP-dependent protein kinase (type II from bovine heart) was prepared and stored as previously described (Peters et al., 1977). The heat-stable protein kinase inhibitor was purified to homogeneity from rabbit skeletal muscle as already described (Demaille et al., 1977) and used without prior separation of the isoinhibitors (Ferraz et al., 1979). Calmodulin was isolated from bovine brain according to the protocol of Teo et al. (1973), as modified by Walsh & Stevens (1978). Glycogen phosphorylase *b* kinase was prepared from rabbit skeletal muscle according to Cohen (1973). Calmodulin-dependent myosin light-chain kinase was isolated from bovine heart as previously described (Walsh et al., 1979). Canine cardiac phosphoprotein phosphatase S was prepared according to Li et al. (1978) through the ethanol and ammonium sulfate precipitation steps. The protein inhibitor of cAMP-independent protein kinase (Job et al., 1979) was a generous gift from Dr. E. Chambaz, Grenoble. Glycogen phosphorylase *b* was isolated from rabbit skeletal muscle according to Fischer & Krebs (1958).

Methods

Miscellaneous Methods. Protein concentrations were determined by the Lowry procedure (Lowry et al., 1951) or by the Coomassie blue technique (Spector, 1978). Dodecyl sulfate (0.1%)–polyacrylamide (15% or 5–20% gradient) gel electrophoresis was performed according to Laemmli (1970). Orthophosphate was measured, according to the required degree of sensitivity, by the Fiske & Subbarow technique (Kuttner & Lichtenstein, 1930), by the malachite green method (Itaya & Michio, 1966), or, when [γ-^{32}P]ATP was used as a substrate of the Ca^{2+}–Mg^{2+}–ATPase, by the paper technique of Reimann & Umfleet (1978).

Preparation of Canine Cardiac SR Vesicles. SR vesicles were prepared from dog hearts arrested in diastole after pentobarbital injection, essentially using the protocol described by Arrio et al. (1974) for skeletal muscle, with the following modifications. Cardiac muscle (60 g) was homogenized for 30 s in a Sorvall Omnimixer operated at full speed in 400 mL 0.1 mM PMSF, 1 mM DTT, 0.3 M sucrose, and 10 mM Hepes buffer, pH 7.5 (buffer A). A major feature of the protocol was the overnight incubation in buffer A containing 0.6 M KCl followed by 3 cycles of washing and sedimentation in this buffer. The final pellet was resuspended in buffer A and used within 12 h or stored frozen under nitrogen. The final yield was 1 mg of SR protein per g of ventricular tissue. Homogeneity of the preparation was assessed by electron microscopy after negative staining, by dodecyl sulfate–polyacrylamide gel electrophoresis, and by measurement of the following enzyme activities: cytochrome *c* oxidase, 5′-nucleotidase, and K^+–EDTA–myosin–ATPase (Bergmeyer, 1974).

The Ca^{2+}–Mg^{2+}–ATPase of the SR vesicles (60 μg of protein per mL) was measured in a 5 mM ATP or [γ-^{32}P]ATP, 10 mM Mg^{2+}, 0.1 mM Ca^{2+}, 0.15 M KCl, 5 mM oxalate, 0.1 mM phosphate, and 10 mM Hepes buffer, pH 7.2, at 20 °C. The rate of calcium uptake was determined at 20 °C essentially according to Martonosi & Feretos (1964) in a 5 mM oxalate, 150 mM KCl, 0.1 mM $^{45}CaCl_2$ (10000–50000 cpm/mL), 5 mM ATP, 10 mM Mg^{2+}, and 10 mM Hepes buffer, pH 7.2, containing 60 μg of SR protein per mL. Aliquots (0.2 mL) were withdrawn at 1-min intervals and filtered through Millipore GSWP 0.22-μm filters previously soaked in 2 mg/mL BSA. Filters were washed twice with 1 mL of 0.1 M $CaCl_2$ and counted in 10 mL of dioxane–naphthalene scintillant.

SR phosphorylations were carried out at 20 °C in a 0.05 mM DTT, 10 mM NaF, and 30 mM sodium phosphate buffer, pH 7.0, containing 0.6 mg of SR protein per mL and either 0.5 μM of the catalytic subunit of cAMP-dependent protein kinase (specific activity 1.16 μmol of ^{32}P incorporated per min per mg at 30 °C) or 0.5 μM calmodulin. Either 0.5 mM Ca^{2+} or 5 mM EGTA was added. The reaction was initiated by addition of 10 mM Mg^{2+} and 0.3 mM [γ-^{32}P]ATP (100–500 cpm/pmol). Aliquots (10 μL) were withdrawn at different times and added to 0.1 mL of 10 mM ATP and 10 mg/mL BSA. Proteins were immediately precipitated by 1 mL of 2 mM ATP and 15% trichloroacetic acid, centrifuged, redissolved at 0 °C in 0.1 mL of 0.1 N NaOH, and immediately precipitated again by 1 mL of 2 mM ATP and 15% trichloroacetic acid. This operation was repeated twice. Finally, the protein pellet was dissolved in 0.5 mL of 98% formic acid, and 0.4 mL of this solution was counted in 10 mL of dioxane–naphthalene scintillant. In some experiments, glycogen phosphorylase *b* (25 μM of the monomer with M_r 96000) was added as an exogenous substrate. Phosphorylation of SR membranes was also carried out in the presence of calmodulin-dependent myosin light-chain kinase (1.5 μM) at pH 8.0 or of phosphorylase *b* kinase (0.12 μM of the $\alpha,\beta,\gamma,\delta$ M_r 335000 complex) at pH 8.2, either in the native state or denatured by a 3-min heat step at 100 °C. The cAMP-dependent protein kinase inhibitor (18.5 nM) or the cAMP-independent protein kinase inhibitor (10 I_{50}) (Job et al., 1979) was added to observe their effect, if any.

Enzymatic Removal of Bound Phosphate. Enzymatic removal of bound phosphate was achieved through incubation of SR vesicles (0.6 mg/mL) in 0.2 mL of 1 mM DTT and 50 mM Hepes buffer, pH 7.4, at 20 °C in the presence of an amount of phosphoprotein phosphatase S that catalyzes the hydrolysis of 0.5 nmol of P_i from a phosphohistone mixture per min. Alternatively, 10 μg of potato acid phosphatase (specific activity 60 units/mg) was preincubated for 1 h at room temperature with 20 μL of 10 mg/mL BSA and added to 0.2 mL of a SR vesicle suspension (0.6 mg/mL) in 0.1 M ammonium acetate buffer, pH 5.5, at 20 °C.

In both cases, 10-μL aliquots were withdrawn and filtered through GSWP Millipore filters (0.22 μm) saturated with BSA. Filters were washed twice with 1 mL of 2 mM ATP and 50 mM potassium phosphate, pH 7.0, and counted in 3 mL of dioxane–naphthalene scintillant.

Calmodulin Iodination. For protection of its ability to bind to calmodulin-dependent enzymes, calmodulin was iodinated by using the chloramine T procedure (Hunter & Greenwood, 1962) under the form of its ternary complex with Ca^{2+} and myosin light-chain kinase, i.e., 7.5 μg of kinase-bound calmodulin in 10 μL of 10 μM Ca^{2+} and 0.25 M potassium phosphate buffer, pH 7.5. After labeling, excess ^{125}I was separated by gel filtration on Sephadex G-50 equilibrated with 1% (w/v) ammonium bicarbonate. [^{125}I]Calmodulin was then separated from myosin light-chain kinase on Sephacryl S-200 equilibrated with 2 mM EDTA and 12.5 mM phosphate buffer, pH 7.5, containing 2 mg of BSA per mL.

The calmodulin peak (specific activity ≥120000 cpm/pmol) was identified by coelectrophoresis with unlabeled calmodulin on dodecyl sulfate–polyacrylamide gel. Its ability to bind to calmodulin-dependent enzymes was checked by gel filtration of a mixture of [^{125}I]calmodulin (60000 cpm) and myosin

light-chain kinase (120 μg) on a Sephadex G-100 column equilibrated with 5 mM Ca^{2+} and 10 mM Tris buffer, pH 7.5, containing 0.5 mg of BSA per mL. Under these conditions, 59% of the radioactivity emerged with the high molecular weight peak corresponding to the ternary complex of Ca^{2+}–calmodulin–kinase.

Calmodulin binding to SR membranes was measured at 20 °C in 10 mM Mg^{2+}, 0.15 M KCl, and 10 mM Hepes buffer, pH 7.2, containing 1.0 mg of SR protein per mL and [^{125}I]-calmodulin (32000 cpm). Either 8 mM Ca^{2+} or 8 mM EGTA was added. [^{125}I]Calmodulin was displaced by 12 μM unlabeled calmodulin. Free and bound calmodulin was separated by filtration on BSA-saturated Millipore filters (GSWP, 0.22 μm). Filters were washed 4 times with 1 mL of 2 mg/mL BSA solution and counted in a Packard Model A 5105 γ counter.

Results

Canine cardiac sarcoplasmic reticulum vesicles, prepared as described above, were not contaminated by actomyosin since no myosin heavy chain was detectable on the electrophoretograms and no K^+–EDTA–myosin–ATPase activity could be measured. Sarcolemmal and mitochondrial contaminations were small, as judged from electron micrographs and from 5′-nucleotidase [4 nmol/(min mg)] and cytochrome oxidase [100 nmol/(min mg)] activities. Moreover, the Ca^{2+}-stimulated ATPase activity [292 nmol/(min mg)] was almost insensitive to 5 mM azide and to 50 μM oligomycin [255 and 216 nmol/(min mg), respectively]. The initial rate of Ca^{2+} uptake was from 0.3 to 0.45 μmol/(min mg), and Ca^{2+} was accumulated in the presence of oxalate up to 3.5–4 μmol/mg.

By the assumption that the Ca^{2+}–Mg^{2+}–ATPase activity of right-side-out cardiac SR vesicles is 0.81 μmol/(min mg) (Jones et al., 1979), properly oriented vesicles represent ~36% of our preparation, in terms of protein content. If the pump monomer of M_r ~100000 makes up ~90% of SR integral proteins, 1 mg of SR protein would correspond to ~320 μg of right-side-out ATPase monomer, i.e., 3.2 nmol.

cAMP-Dependent Phosphorylation of SR. When SR vesicles, prepared as described under Methods, were incubated in the presence of 15 μM cAMP or in the presence of 0.5 μM purified catalytic subunit of cAMP-dependent protein kinase, phosphate was mostly incorporated into phospholamban (M_r 22000) and into two fainter bands of M_r 11000 and 6000. Excess protein kinase inhibitor abolished phosphate incorporation. Addition of Triton X-100 to the samples prior to dodecyl sulfate gel electrophoresis dramatically altered the phosphorylation pattern. The M_r 22000 band disappears, and the radioactivity migrates essentially with a velocity corresponding to M_r 11000. The intensity of the M_r 6000 band is unchanged.[2] There is evidence that the mixture of ionic and nonionic detergents results in the dissociation of a dimeric M_r 22000 species into two presumably identical polypeptide chains of M_r 11000 (C. J. Le Peuch, D. A. M. Le Peuch, and J. G. Demaille, unpublished experiments).

Experiments were undertaken to assess the extent of phosphate incorporation catalyzed by the catalytic subunit, which proceeded rapidly up to a plateau of 3.25 nmol/mg of SR protein (Figure 1). If the assumptions above (i.e., 3.2 nmol of ATPase monomer per mg of SR protein) stand true, our data are compatible with a model in which the phospholamban monomer is present in 1:1 stoichiometric amounts compared to the ATPase monomer and exhibits a single discrete site of phosphorylation by cAMP-dependent protein kinase. In this case, the phospholamban content of SR vesicles would amount to one-tenth (w/w) that of the ATPase content, even though phospholamban is difficult to stain with Coomassie blue.

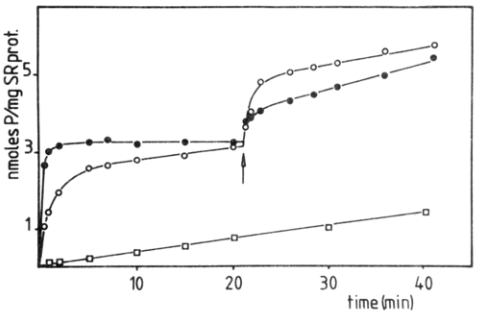

FIGURE 1: Phosphorylation of cardiac sarcoplasmic reticulum vesicles. SR vesicles were incubated, as described under Methods, in the presence of 5 mM EGTA and 18.5 nM protein kinase inhibitor (□). Alternatively, 0.5 mM Ca^{2+} and 0.5 μM catalytic subunit of cAMP-dependent protein kinase were added at zero time, followed by addition of 0.5 μM calmodulin at the time indicated by the arrow (●). In another experiment, Ca^{2+} and calmodulin were added first, followed by the catalytic subunit at the time indicated by the arrow (○).

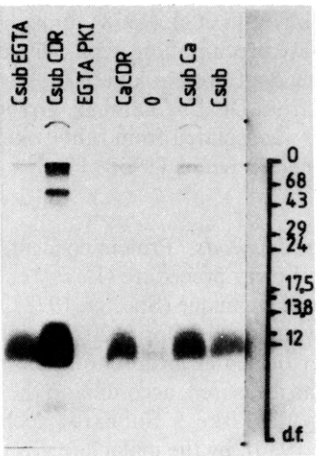

FIGURE 2: Autoradiogram of a 0.1% dodecyl sulfate–15% polyacrylamide gel electrophoretogram of SR vesicles phosphorylated under different conditions. (C sub EGTA) 0.5 μM catalytic subunit of cAMP-dependent protein kinase and 5 mM EGTA; (C sub CDR) 0.5 μM catalytic subunit, 0.5 mM Ca^{2+}, and 0.5 μM calmodulin; (EGTA PKI) 5 mM EGTA and 18.5 nM protein kinase inhibitor; (Ca CDR) 0.5 mM Ca^{2+} and 0.5 μM calmodulin; (0) no addition; (C sub Ca) 0.5 μM catalytic subunit and 0.5 mM Ca^{2+}; (C sub) 0.5 μM catalytic subunit. Other conditions are as described under Methods. When the phosphorylation plateau was reached (see Figure 1), samples were withdrawn, mixed with the 1% dodecyl sulfate and 1% Triton X-100 sample buffer, and boiled for 5 min. (O) Origin; (d.f.) dye front. The figures represent the molecular weights ($\times 10^3$) of the following markers: bovine serum albumin, ovalbumin, carbonic anhydrase, trypsinogen, β-lactoglobulin, lysozyme, and parvalbumin. The migration of myoglobin and cytochrome *c* is also indicated.

The phosphorylation by the C subunit was not Ca^{2+} dependent, as shown in Figure 2. Addition of 0.5 mM Ca^{2+} resulted in a small inhibition of the cAMP-dependent phosphorylation[2] which can be ascribed to both a well-known inhibitory effect of Ca^{2+} on the kinase (Rubin et al., 1974) and to a rapid depletion of the ATP by the calcium pump.

Calmodulin-Dependent Phosphorylation of SR. In the absence of cAMP or the catalytic subunit of cAMP-dependent protein kinase and in the presence of 5 mM EGTA and protein kinase inhibitor, the rate of phosphorylation of SR vesicles was ~0.035 nmol/(min mg of SR protein). Addition of 0.5 mM Ca^{2+} and 0.5 μM calmodulin resulted in a rapid phosphorylation of SR up to the same level of incorporation reached with

[2] The data are not illustrated, but were submitted to reviewers for examination. The material will be sent to interested readers upon request.

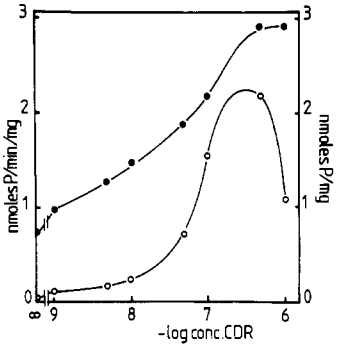

FIGURE 3: Initial rate of the calmodulin-dependent phosphorylation of SR vesicles and level of the phosphorylation plateau. SR vesicles (0.6 mg/mL) were phosphorylated in the presence of 0.5 mM Ca^{2+} and variable concentrations of calmodulin as described under Methods. The initial rate (○, nmol of P per min per mg) was taken from the first 30 s of incubation. The plateau value (●, nmol of P per mg) was obtained after 45 min.

the catalytic subunit (see Figure 1). Phosphate was, in this case too, essentially incorporated into phospholamban (see Figure 2). The possibility that the same site(s) that is (are) phosphorylated by the catalytic subunit of cAMP-dependent protein kinase is (are) involved in the calmodulin-dependent phosphorylation was ruled out by experiments in which SR vesicles were incubated in the presence of Ca^{2+} with the C subunit first and then with calmodulin or with calmodulin first and then with the C subunit (see Figure 1).

In both types of experiments, phosphorylation resumed after addition of calmodulin or the catalytic subunit to reach 5.5–5.8 nmol of ^{32}P per mg of SR protein after ~40 min of incubation, i.e., 1.7–1.8 phosphate groups/phospholamban monomer of M_r 11 000. It is thus reasonable to conclude that the phospholamban monomer exhibits two stoichiometric sets of phosphorylation sites which are substrates for cAMP-dependent protein kinase and a calmodulin-dependent protein kinase present in the SR vesicles preparation, respectively.

Whether calmodulin was added first or after the C subunit, the calmodulin-dependent phosphorylation, even at the most favorable calmodulin concentration, proceeded at a slower rate than the one catalyzed by the C subunit under similar conditions (see Figure 1). No definite conclusion can be drawn, however, as to what happens in vivo. The concentration of the catalytic subunit used in the above experiments (0.5 μM) is close to the maximal concentration (0.38 μM) (Hofmann et al., 1977) reached upon full dissociation of the protein kinase holoenzymes. By contrast, it is not known whether part of the calmodulin-dependent kinase was lost during purification or whether maximal velocity was obtained under our experimental (pH and ionic strength) conditions. At any rate, phosphorylation by one or the other kinase clearly results in a slower rate of phosphorylation at the other site, as shown in Figure 1.

Properties of the Calmodulin-Dependent Protein Kinase. The calmodulin-dependent protein kinase was totally dependent on Ca^{2+} since no incorporation of phosphate occurred in the presence of EGTA, irrespective of the calmodulin concentration.[2] It is also totally dependent upon calmodulin, as shown by the increase in the initial phosphorylation rate in the presence of increasing amounts of calmodulin (Figure 3). In the absence of exogenous calmodulin, the rate of phosphorylation in the presence of Ca^{2+} is very slow [<60 pmol/(min mg)] and could be attributed to a contamination of the SR preparation by less than 1 nM calmodulin.

Under our experimental conditions (0.6 mg of SR protein per mL), half-maximal initial rate of phosphorylation was reached at 70 nM calmodulin and maximal initial rate at 0.5 μM. Above this concentration, the rate of reaction decreased again.

However, the same plateau was still obtained in the presence of 1 μM as well as of 0.5 μM calmodulin. Lower concentrations of calmodulin resulted in lower levels of the phosphorylation plateau. Half-maximal final incorporation was reached at the same calmodulin concentration that induced half-maximal initial reaction velocity. Together with the fact that the kinase is still present in 0.6 M KCl washed SR vesicles, the latter fact argues for a strong association of the kinase with or within the membrane (Figure 3).

The presence of calmodulin binding sites on SR membranes was verified by using [^{125}I]calmodulin. Binding occurred within the first minute and was stable over 30 min. After correction for nonspecific binding (i.e., the radioactivity of the filter in the absence of SR vesicles), the ratio total radioactivity/bound radioactivity is equal to $1 + K_d/[\text{total kinase}]$, in which K_d is the dissociation constant of the calmodulin–kinase complex. In the presence of Ca^{2+}, $K_d/[\text{total kinase}] = \sim 6$. Excess unlabeled calmodulin abolished the binding of [^{125}I]calmodulin in the presence of Ca^{2+}.

The presence of calmodulin binding sites on SR membranes was also checked independently by addition of [^{125}I]calmodulin to the cardiac muscle homogenate at the beginning of the SR preparation. In the first SR pellet, 3% of the initial radioactivity was still present. From this SR-bound calmodulin, 60, 7, and 1% were removed in the three sequential washes by 0.6 M KCl.

The calmodulin-dependent phosphorylation of phospholamban was not sensitive to the inhibitor of cAMP-dependent protein kinase or to the inhibitor of cAMP-independent protein kinases.[2]

The K_m for ATP of the calmodulin-dependent kinase appears to be substantially higher than the one exhibited by the C subunit [13 μM (Rubin et al., 1974)] since ATP addition during the course of phosphorylation, designed to prevent ATP exhaustion by the pump ATPase, always resulted in a transient acceleration of the phosphorylation reaction that was not observed with the C subunit. Although no precise figure can be given since ATP was being split at the same time by the pump ATPase, it is reasonable to assume that the K_m for ATP is of the same order of magnitude as the initial ATP concentration (0.3 mM). Other calmodulin-dependent protein kinases, e.g., myosin light-chain kinase (Walsh et al., 1979) or glycogen phosphorylase *b* kinase (Krebs et al., 1964), exhibit high K_m values for ATP.

The calmodulin-dependent protein kinase is labile. Although phospholamban phosphorylation was evident when the experiment was performed on fresh SR, freezing at 77 K under nitrogen resulted in the loss of 50% of the activity. Similarly, calmodulin binding decreased very rapidly and disappeared after 24 h of storage at 4 °C under nitrogen. Slow freezing at −20 °C under nitrogen afforded the best storage conditions. In some experiments, the requirement for Ca^{2+} and calmodulin disappeared upon storage and the kinase activity increased.[2] Since, during the same time, the ATPase band of M_r 100 000 also disappeared, it is likely that a regulatory subunit or domain was destroyed by contaminating proteases. Such proteolytic activation has been described for phosphorylase *b* kinase (Krebs et al., 1964; Cohen, 1973). It was accompanied by a loss of Ca^{2+} sensitivity in the calmodulin-dependent phosphodiesterase (Moss et al., 1978) and in the platelet myosin light-chain kinase (Daniel & Adelstein, 1976).

Nature of the Calmodulin-Dependent Protein Kinase. Glycogen phosphorylase *b* kinase contains calmodulin (Cohen

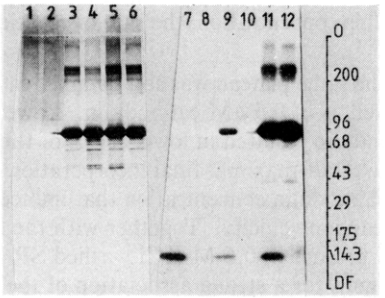

FIGURE 4: Phosphorylation of exogenous glycogen phosphorylase *b* and of phospholamban either by SR vesicles in the presence of calmodulin or by exogenous phosphorylase *b* kinase in the absence of free calmodulin. SR vesicles (0.6 mg/mL) were incubated in a 10 mM Tris buffer, pH 8.2, with the additions mentioned below, 10-μL samples were withdrawn after 45 min and loaded on 0.1% dodecyl sulfate–5 to 20% polyacrylamide gel. The electrophoretogram was stained with Coomassie blue (1–6) and used for autoradiography (7–12). Additions: (1 and 7) 0.5 mM Ca^{2+} and 0.5 μM calmodulin; (2 and 8) 5 mM EGTA; (3 and 9) 25 μM phosphorylase *b*, 0.5 mM Ca^{2+}, and 0.5 μM calmodulin; (4 and 10) 25 μM phosphorylase *b* and 5 mM EGTA; (5 and 11) 25 μM phosphorylase *b*, 0.12 μM phosphorylase *b* kinase, and 0.5 mM Ca^{2+}; (6 and 12) same as for 5 and 11, except that SR vesicles were omitted in this sample. Molecular weight markers are as in Figure 2.

et al., 1978) and has been proposed to be involved in the cAMP-dependent phosphorylation of phospholamban (Wray & Gray, 1977). Also, a calcium-dependent protein kinase that is very similar but not identical to phosphorylase kinase is present in isolated vesicles of skeletal muscle SR and activates the Ca^{2+} transport ATPase (Hörl et al., 1978; Hörl & Heilmeyer, 1978). We therefore checked whether on the one hand phospholamban kinase can phosphorylate glycogen phosphorylase *b* and whether on the other hand glycogen phosphorylase *b* kinase can phosphorylate phospholamban.

As shown in Figure 4, exogenous phosphorylase *b* is phosphorylated in the presence of SR vesicles and calmodulin. The phosphorylation is both Ca^{2+}- and calmodulin-dependent[2] and proceeds much further than would be expected from the sole phosphorylation of phospholamban. Both phosphorylase *b* and phospholamban were shown to be phosphorylated by dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography (Figure 4). The inhibitors of cAMP-dependent and cAMP-independent protein kinases, respectively, did not alter the rate of phosphorylation of phosphorylase *b*.[2] SR vesicles thus exhibit a protein kinase similar to glycogen phosphorylase *b* kinase, if it were not for the requirement for exogenous calmodulin.

Addition of exogenous glycogen phosphorylase *b* kinase in the absence of calmodulin results in a Ca^{2+}-dependent phosphorylation of SR vesicles (see Figure 4) that is almost as fast as the one obtained in the presence of calmodulin and Ca^{2+} alone (Figure 5A). Autophosphorylation of the added enzyme cannot account for the incorporation of phosphate. Since it has been reported than phosphorylase kinase bound calmodulin can activate other calmodulin-dependent enzymes (Cohen et al., 1978), a parallel experiment was carried out by using heat-denatured phosphorylase kinase in which calmodulin (δ subunit) dissociates from the other subunits. Incorporation of phosphate in the presence of the boiled exogenous kinase proceeds much slower and reaches a lower plateau, indicating that the native exogenous phosphorylase kinase catalyzes SR phosphorylation rather than simply activates the membrane-bound kinase through its bound calmodulin (Figure 5A).

In the presence of another calmodulin-dependent enzyme, cardiac myosin light-chain kinase, and of calmodulin, the Ca^{2+}-dependent phosphorylation was also slower and reached

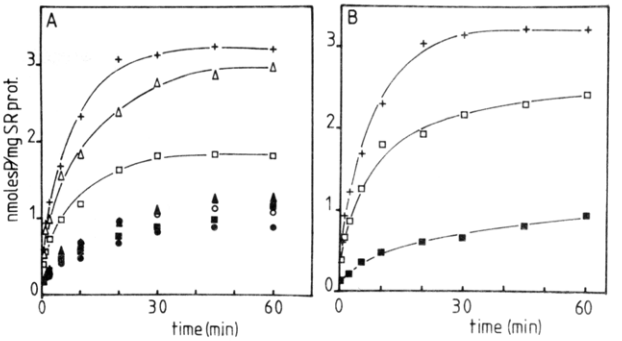

FIGURE 5: Phosphorylation of SR vesicles in the presence of exogenous calmodulin and/or calmodulin-dependent enzymes. (A) 0.5 μM calmodulin and 0.5 mM Ca^{2+} at pH 8.0 (+); 0.12 μM glycogen phosphorylase *b* kinase and 0.5 mM Ca^{2+} at pH 8.2 (△); 0.12 μM heat-denatured phosphorylase *b* kinase and 0.5 mM Ca^{2+} at pH 8.2 (□); 0.12 μM phosphorylase *b* kinase and 5 mM EGTA at pH 8.2 (▲); 5 mM EGTA (●); 0.5 mM Ca^{2+} (○); 0.12 μM heat-denatured phosphorylase *b* kinase and 5 mM EGTA at pH 8.2 (■). (B) 0.5 μM calmodulin and 0.5 mM Ca^{2+} at pH 8.0 (+), redrawn from Figure 5A to help comparison with other curves; 1.5 μM calmodulin-deficient myosin light-chain kinase, 0.5 μM calmodulin, and 0.5 mM Ca^{2+} at pH 8.0 (□); 1.5 μM calmodulin-deficient myosin light-chain kinase, 0.5 μM calmodulin, and 5 mM EGTA at pH 8.0 (■). Other conditions as described under Methods, except for the use of a 10 mM Tris buffer.

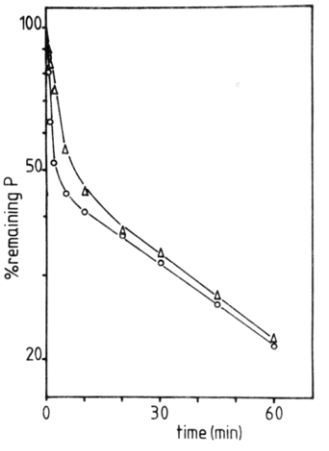

FIGURE 6: Removal of protein-bound phosphate by potato acid phosphatase (○) or by phosphoprotein phosphatase S (△). The experimental procedure was as described under Methods.

a lower plateau than with Ca^{2+}–calmodulin alone. Phospholamban therefore does not appear to be a substrate for the myosin light-chain kinase, which competes with phospholamban kinase for the available calmodulin (Figure 5B).

Effect of Phosphatases on Phosphorylated SR Vesicles. The endogenous phosphatase activity was evaluated from experiments conducted in the presence or absence of 10 mM NaF.[2] The steady-state level of phosphorylation obtained in the absence of NaF was only slightly lower.

For the study of dephosphorylation by exogenous phosphatases, SR vesicles were fully phosphorylated in the presence of both the C subunit of cAMP-dependent protein kinase and 0.5 μM calmodulin–0.5 mM Ca^{2+}. When the vesicles were incubated in the presence of phosphoprotein phosphatase S from canine cardiac muscle (Li et al., 1978), approximately half of the bound phosphate was removed rapidly (k = 0.2/min, $t_{1/2}$ = 3.5 min) whereas hydrolysis of the residual bound phosphate proceeded much more slowly (k = 0.014/min, $t_{1/2}$ = 50 min) (Figure 6). Very similar results were obtained by using potato acid phosphatase, with a first rapid phase (k = 0.46/min, $t_{1/2}$ = 1.5 min) and a slower removal of the second half of the bound phosphate (k = 0.013/min, $t_{1/2}$ = 53 min)

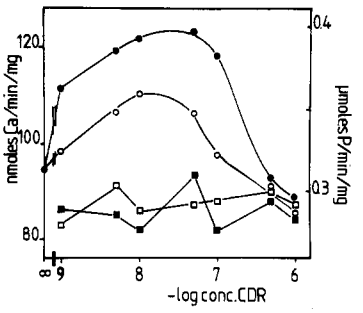

FIGURE 7: Effect of calmodulin and of the catalytic subunit of cAMP-dependent protein kinase on the Ca^{2+}–Mg^{2+}–ATPase activity and on the rate of Ca^{2+} uptake by SR vesicles. SR vesicles (60 μg/mL) were incubated, as described under Methods, in the presence of variable calmodulin concentrations and of either 18.5 nM protein kinase inhibitor (open symbols) or 62 nM catalytic subunit (closed symbols). The rate of calcium uptake (○, ●) and the Ca^{2+}–Mg^{2+}–ATPase activity (□, ■) were measured as described under Methods.

(Figure 6). The intercept of the second slope of the graph with the ordinate was ca. 49 and 46% for phosphoprotein phosphatase and acid phosphatase, respectively. This points either to two sites (or two sets of sites) in a 1:1 stoichiometry with different structures and therefore affinities for the added phosphatase or, as was suggested above for the phosphorylation, to a conformational change after removal of half of the bound phosphate from phospholamban.

Effect of Phosphorylations on the Calcium Uptake and the Ca^{2+}–Mg^{2+}–ATPase Activity. The Ca^{2+}–Mg^{2+}–ATPase activity and the Ca^{2+} uptake rate were measured on the same SR vesicle suspension in the presence of increasing concentrations of calmodulin. Whether the endogenous cAMP-dependent protein kinase activity was inhibited by addition of protein kinase inhibitor or, on the contrary, the C subunit was added to the reaction mixture, no significant modification of the ATPase activity was observed in the presence of calmodulin at several concentrations (from 10^{-9} to 10^{-6} M) (Figure 7). Under the same conditions, calmodulin alone, in the presence of protein kinase inhibitor, induced a slight activation of the rate of Ca^{2+} uptake [from 94 to 109 nmol/(min mg) at 10 nM calmodulin; i.e., a 116% activation in this particular preparation]. The rate of Ca^{2+} uptake then decreased slowly to fall below the rate observed in the absence of calmodulin, at 0.5 μM calmodulin, and above.

In the presence of the catalytic subunit of cAMP-dependent protein kinase, a similar activation was obtained but with a greater amplitude, the rate of Ca^{2+} uptake reaching 118 nmol/(min mg), i.e., a 125% activation above basal level at 50 nM calmodulin. Higher concentrations (0.5 and 1 μM) resulted in an inhibition of the calcium pump similar to that observed in the absence of the catalytic subunit (Figure 7).

These experiments were performed at an SR concentration 10 times smaller than the experiments on phosphorylation. One would expect to need 10 times less calmodulin in the Ca^{2+} uptake assay to activate a similar proportion of the kinase only if the K_d of the calmodulin–kinase complex were ~1 nM and the amount of kinase in the phosphorylation test were ~140 nM (and 14 nM in the Ca^{2+} uptake assay), taking into account 70 nM calmodulin for half-maximal activation of SR phosphorylation.

As the right-side-out ATPase monomer concentration in the phosphorylation test is 1.95 μM, one would thus expect ~1 mol of kinase to be present per 14 mol of ATPase monomer or phospholamban monomer. This stoichiometry is in agreement with the observed levels of the phosphorylation plateaus as a function of the calmodulin concentration.

On the other hand, the estimated concentration of the kinase in the binding experiments (~0.25 μM) and the observed ratio K_d/[total kinase] = ~6 point to $K_d \simeq 1.5$ μM. Labeled calmodulin thus bound poorly to the enzyme. This explains the difficulties encountered in obtaining binding data.

Discussion

Phospholamban, which has been described as an M_r 22000 phosphoprotein, appears in fact to be a dimer of two polypeptide chains of M_r 11000.[3] The existence of an M_r 11000 phosphorylatable polypeptide has already been observed (Kirchberger & Tada, 1976), but it was not suggested that this may be a monomeric form of phospholamban.

It is reasonable to assume that the very hydrophobic chain of ~90 residues is for a large part embedded in the lipid bilayer since the purified monomer exhibits some characteristics of a proteolipid (Bidlack & Shamoo, 1979; C. J. Le Peuch and J. G. Demaille, unpublished experiments). Only a small part of the chain is likely to be accessible to protein kinases and ATP; this is also suggested by the fact that phosphopeptides liberated by digestion of phosphorylated SR vesicles by trypsin, chymotrypsin, subtilisin, or staphylococcal protease are not precipitable by trichloroacetic acid (C. J. Le Peuch and J. G. Demaille, unpublished experiments). Since this short stretch is phosphorylated to the same extent by cAMP-dependent and -independent kinases, each monomer is likely to exhibit a single phosphorylation site by the catalytic subunit of cAMP-dependent protein kinase and 1 mol of phospholamban monomer to be found per mol of ATPase monomer, as was already suggested (Kirchberger & Tada, 1976).

If this were true, phospholamban would represent ~10% of the ATPase content (w/w). It must be practically devoid of lysine residues since it is not stained by Coomassie blue. It is likely that a few arginyl residues, responsible for the tryptic cleavage of phosphopeptides and for the specificity of the substrate site of cAMP-dependent phosphorylation, form the bulk of the basic residues of the molecule.

The phosphorylation of phospholamban by the catalytic subunit of cAMP-dependent protein kinase is a direct event and does not involve the metabolic cascade suggested by Wray & Gray (1977). Using the pure catalytic subunit, we have confirmed indeed that phosphorylation is not Ca^{2+} dependent, as already suggested by Kirchberger et al. (1974).

On the other hand, phospholamban is also a substrate for a calmodulin-dependent kinase, at a phosphorylation site distinct from the site which is a substrate of cAMP-dependent kinase. Katz & Remtulla (1978) have already reported the stimulation of Ca^{2+} transport in cardiac SR vesicles by calmodulin and suggested separate mechanisms of action. In fact, two different kinase systems are involved in the phosphorylation of the same phospholamban molecule. Taking into account the probability that only a short stretch of the chain is accessible, as discussed above, one may envision this stretch as a double-headed peptide substrate forming a β turn and presenting two serine residues on both sides of an arginyl residue. Such a peptide would be similar to the peptide L-S-Y-R-G-Y-S-L that is phosphorylated on the proximal and distal serine residues by phosphorylase kinase and cAMP-dependent protein kinase, respectively (Graves et al., 1978).

[3] The factors responsible for the incomplete dissociation of the M_r 22000 species in the presence of dodecyl sulfate will be discussed in a later paper (C. J. Le Peuch and J. G. Demaille, unpublished experiments) devoted to the purification of the phospholamban monomer.

Purified phospholamban is indeed only phosphorylated on serine residues (C. J. Le Peuch and J. G. Demaille, unpublished experiments).

The Ca^{2+}–calmodulin-dependent protein kinase is obviously firmly bound to the SR membrane since it is still present after three washes by 0.6 M KCl. It has been shown (Moeschler et al., 1979) that the soluble phosphorylase kinase content of SR vesicles prepared from skeletal muscle could be reduced to negligible amounts by successive washes.

The membrane-bound kinase has several features in common with glycogen phosphorylase *b* kinase. Firstly, it is capable of phosphorylating glycogen phosphorylase *b*; also, the exogenous phosphorylase *b* kinase is able, in the absence of free calmodulin, to introduce phosphate into phospholamban at a rate and level beyond those obtained with its calmodulin content alone, as shown with the heat-denatured enzyme. The membrane-bound enzyme, however, differs strikingly from the soluble phosphorylase kinase by its absolute requirement for calmodulin. Calmodulin would not dissociate completely from the other subunits of soluble phosphorylase kinase in the presence of 0.6 M KCl (Cohen, 1979).

A membrane-bound Ca^{2+}-dependent protein kinase that is very similar to phosphorylase kinase has been reported to be present in isolated SR vesicles from rabbit and mouse skeletal muscle and to regulate the Ca^{2+} transport of the SR (Hörl et al., 1978; Hörl & Heilmeyer, 1978; Varsányi et al., 1978). Apparently, this kinase is also different from the one described here since it does not require added calmodulin.

In the absence of any phosphorylation by the cAMP-dependent protein kinase, calmodulin-dependent phosphorylation is able, per se, to induce some activation of the SR calcium uptake. As the calmodulin content of heart (Grand et al., 1979) is not likely to be a limiting factor, this phosphorylation is dependent only upon the concentration of free Ca^{2+} ions.

The cAMP-dependent phosphorylation does not activate Ca^{2+} uptake in the absence of calmodulin but amplifies the activation brought about by the calmodulin-dependent phosphorylation. This accounts for the fact that weak or null activation of Ca^{2+} uptake was observed in the absence of calmodulin. The activations reported in the literature (Kirchberger & Tada, 1976) to occur in response to the cAMP-dependent pathway alone were obtained on cardiac microsomes that were not washed repeatedly in the presence of high salt and therefore contained a significant amount of bound calmodulin. There is no explanation as of now as to the inhibition of Ca^{2+} uptake and of the phosphorylation rate that occurs in the presence of excess calmodulin.

The overall regulation of the pump can tentatively be described as follows. The pump is probably on oligomer (Dean & Tanford, 1978) in close contact with a phospholamban oligomer, perhaps by association of monomers of each protein. Free cytosolic Ca^{2+} modulates the phosphorylation of phospholamban at the calmodulin-dependent site, and the corresponding kinase can be described as a sensor of the outside calcium concentration. The conformational change induced by the phosphoryl residue on the phospholamban chain in its protruding outside part may result in a modification of the hydrophobic microenvironment of the ATPase molecule, as suggested by Chiesi (1979). Introduction of another phosphoryl group, under the influence of epinephrine and the cAMP-dependent pathway, will increase the conformational change and the resultant perturbation of the hydrophobic environment, thereby enhancing the calcium uptake activity.

The ATPase activity of the pump does not appear to be altered in both cases, although the presence of carrier orthophosphate in the experiment precludes any definite conclusion about the apparently improved Ca^{2+}/ATP stoichiometry.

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Consequences of Reacting the Thiols of Myosin Subfragment 1†

Jean Botts,* Kathleen Ue, Tetsu Hozumi,‡ and Jeffrey Samet§

ABSTRACT: When subfragment 1 (S-1) of myosin is exposed (120 h at 0 °C) to a series of increasing concentrations of methyl methanethiosulfonate (MMTS) up to 25 × [S-1], the accessible thiols of S-1 are progressively reacted to form $-SSCH_3$. If "SH_1", the more readily reactive thiol of S-1, is first labeled with the fluorescent dye 1,5-IAEDANS [*N*-(iodoacetyl)-*N*-(5-sulfo-1-naphthyl)ethylenediamine], seven additional thiols can be reacted with MMTS. In 8 M urea 12 thiols of S-1 are titrated with PCMB [*p*-(chloromercuri)benzoate]. Thus, four thiols are considered to be "inaccessible" to MMTS under the exposure conditions used. S-1 ATPase activities (Ca^{2+}, Mg^{2+}, and actin activated) decline as more MMTS is bound but do not fall to zero even at [MMTS]/[S-1] ratios up to 100:1. With fluorescent-labeled S-1, the affinity of S-1 for F-actin is measured by following the time-resolved fluorescence anisotropy decay. As more MMTS is bound to S-1, the affinity constant decreases from about 10 μM^{-1} to about 2 μM^{-1} (25 °C; 0.284 M KCl, 1.35 mM $MgCl_2$, and 0.45 mM EGTA, buffered at pH 7.4). In the presence of ATP there is no measurable affinity. Since reacting S-1 thiols with a small blocking group such as MMTS does not abolish S-1 ATPase activity or actin binding, it is concluded that thiols are not directly involved in these functions. The effects of reacting S-1 thiols with MMTS may be attributed to conformational changes in S-1. To test this hypothesis, we applied certain probes of structural changes at different stages of blockage with MMTS. The fluorescence of 8-anilino-1-naphthalenesulfonate equilibrated with the system increased with increased blockage but not hand in hand with the ATPase activity. However, signals (emanating from 1,5-IAEDANS placed at thiol SH_1) from two other probes correlated very well with loss of ATPase activity and with loss of actin affinity, and their behavior can be taken to mean that certain structural changes are progressively caused by progressive blockage. The rotational correlation time of the S-1 particle increases, suggesting a progressive global deformation. The magnitude of the excited-state lifetime decreases upon ATP addition, suggesting a progressive accessibility of solvent to the region immediately near to SH_1.

Pursuing our laboratory's long-standing interest in the possibility that "activation" and "inhibition" (collectively, "modification") of myosin ATPase are conformational phenomena rather than a consequence of thiol reaction per se (Rainford et al., 1964; Hartshorne & Morales, 1965), we undertook a study of the effects of reacting myosin thiols using a "small" SH reagent, with the idea of minimizing *indirect* effects of thiol reaction, e.g., results of putting bulky groups at structurally sensitive locations. Our experiments were designed to examine also the essentiality of thiols to the myosin affinity for actin. Finally, we included in our design optical probes that might sense conformational changes if these occurred. The consequences of reacting the thiols of myosin S-1[1]

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‡ T.H. is a Fellow of the American Heart Association.
§ Present address: Baylor College of Medicine, Houston, TX.

[1] Abbreviations used: S-1, myosin subfragment 1; SH_1, fast-reacting thiol in S-1; 1,5-IAEDANS, *N*-(iodoacetyl)-*N*-(5-sulfo-1-naphthyl)-ethylenediamine; MMTS, methyl methanethiosulfonate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PCMB, *p*-(chloromercuri)benzoate; DTT, dithiothreitol; Ans, 8-anilino-1-naphthalenesulfonate; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; IAA, iodoacetamide.

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