Immunological Detection of Phospholamban Phosphorylation States Facilitates the Description of the Mechanism of Phosphorylation and Dephosphorylation†

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Received September 6, 1989; Revised Manuscript Received January 22, 1990

ABSTRACT: Six electrophoretically distinct species of oligomeric phospholamban were identified immunologically following phosphorylation of sarcoplasmic reticulum vesicles by cAMP-dependent protein kinase. The phosphate content of each was determined, confirming that the discrete sequential retardation of phospholamban oligomers was the result of ascending mole ratios of phosphate (P_0–P_5) per oligomer. These data afford support to the pentameric arrangement of oligomeric phospholamban and offer a means of determining phosphorylation stoichiometry independent of the absolute phospholamban concentration. Detection of the relative concentration of individual species during phosphorylation facilitated the description of a random mechanism of phosphorylation by cAMP-dependent protein kinase. By contrast, dephosphorylation of cAMP-dependent protein kinase phosphorylated phospholamban was shown to exhibit strong positive cooperativity in its reaction mechanism.

Cardiac contractility can be regulated by circulating catecholamines. β-Adrenergic stimulation of the cardiac myocyte results in an elevation of the adenosine cyclic 3′,5′-monophosphate (cAMP)[1] concentration, activation of cAMP-dependent protein kinase, and phosphorylation of, among other targets, a sarcoplasmic reticulum protein called phospholamban (Tada & Katz, 1982). Phosphorylation of phospholamban is associated with an increase in the rate of calcium accumulation by SR, an effect mediated through stimulation of the calcium pump, (Ca^{2+}–Mg^{2+})-ATPase (Tada et al., 1982; Gupta et al., 1988; Kirchberger et al., 1986).

Two forms of phospholamban can be identified functionally or immunologically (Suzuki & Wang, 1986; Jones et al., 1985) following electrophoresis on SDS–PAGE. These forms correspond to a monomeric form of 5–6 kDa and a polymeric unit of 22–29 kDa (Suzuki & Wang, 1986; Jones et al., 1985). Inaccuracies inherent in the molecular weight determination of membrane proteins by electrophoresis (Ragan, 1986) have impeded the description of phospholamban oligomer compositions by these criterion alone. Heat treatment of the SR sample, however, can convert the oligomeric structure into the monomeric form, and several groups have applied controlled exposure to heat to describe the dissociation of radiolabeled oligomer. Five separate radiolabeled species are observed on dissociation of the oligomer (Wegener & Jones, 1984; Fujii et al., 1987), prompting interpretation of a pentameric arrangement (Wegener et al., 1986; Simmerman et al., 1986). This conclusion was supported by studies designed to exploit the electrophoretic mobility changes of phospholamban following phosphorylation. The oligomeric form of phospholamban has been shown repeatedly to migrate at a higher apparent molecular weight than nonphosphorylated forms (Suzuki & Wang, 1986; Wegener & Jones, 1986), and this was resolved into a series of phosphorylated intermediates by Imagawa et al. (1986). Five phosphorylated species of oligomeric phospholamban were detected autoradiographically, consistent with the pentameric model.

The functional significance of this pentameric arrangement is unknown. In the present study, we are also able to confirm the pentameric organization of phospolamban by immunological detection of the phosphorylation species. This approach, which allows the specific visualization of phospholamban, can be used to address the mechanism of phosphorylation of phospholamban while still in the environment of an intact SR membrane. This methodology has certain advantages over alternative strategies employing purified protein, since it is conceivable that the purified phospholamban behaves differently to that in SR. Detection of the relative protein concentration of each phosphorylated species by immunological means (including the nonphosphorylated form) has facilitated the description of the mechanism of phosphorylation and dephosphorylation. Cyclic AMP dependent protein kinase is shown to phosphorylate phospholamban following a random (noncooperative) mechanism, whereas dephosphorylation of such a phosphoprotein by endogenous phosphatases follows a strictly cooperative pathway.

Experimental Procedures

Materials. [γ-^{32}P]ATP (10 mCi/mmol) was supplied by ICN Radiochemicals (Irvine, CA), and other chemicals were from Sigma and Bio-Rad. The catalytic subunit of bovine cardiac cyclic AMP dependent protein kinase was prepared by an established procedure (Peters et al., 1977). Canine cardiac sarcoplasmic reticulum was purified according to the method of Chamberlain et al. (1983), although the sucrose

† This work was supported by an operating grant from the Canadian Heart Foundation.

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[1] Abbreviations: SR, sarcoplasmic reticulum; ATPase, adenosinetriphosphatase; cAMP, adenosine cyclic 3′,5′-monophosphate; PKI, protein kinase A inhibitor peptide 5–24 amide; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; TBS, Tris-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; P_LL, monomeric phospholamban; P_LH, oligomeric phospholamban; ATP, adenosine 5′-triphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); Tris, tris(hydroxymethyl)aminomethane; A-kinase, cAMP-dependent protein kinase.

0006-2960/90/0429-4535$02.50/0

gradient centrifugation procedure was omitted in these studies. Monoclonal antibody A1, directed against canine cardiac phospholamban (Suzuki & Wang, 1986), was purified by hydroxylapatite chromatography as described by Stanker et al. (1985).

SDS–PAGE and Immunoblotting. Twenty micrograms of SR protein was solubilized for 30 min at 30 °C and resolved on 15% acrylamide gels (160 × 140 × 0.75 mm) as described by Laemmli (1970). Proteins were transferred to Zeta-Probe membranes as described by Towbin et al. (1979), overnight at 10 V or for 4 h at 55 V, 4 °C.

Membranes were incubated with 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 2% dried milk powder (TBS–milk) for 30 min prior to addition of mAb A1, 2.5 μg/mL, in TBS–milk. Antibody was removed after 3 h at room temperature and the membrane incubated with goat anti-mouse IgG peroxidase (1:5000 dilution in TBS–milk, Jackson) for a further 3 h. Finally, the membrane was incubated for 10 min with substrate (2.9 mM 4-methoxy-1-naphthol in TBS with 0.027% H_2O_2). Densitometric analysis of the resolved oligomeric species was performed on an LKB 2202 Ultrascan, and peak areas were integrated by using an interfaced Hewlett Packard 3390A integrator.

Phosphorylation by the Catalytic Subunit of Cyclic AMP Dependent Protein Kinase. Phosphorylation of SR membranes was performed essentially as described in Suzuki and Wang (1987), using 0.15 mM [γ-^{32}P]ATP (100–1500 cpm/pmol) and 0.25 μg of catalytic subunit of cAMP-dependent protein kinase (final volume 200 μL) at 30 °C in the absence of calcium ions (0.1 mM EGTA). The reaction was initiated by the addition of ATP and terminated by the addition of SDS–sample buffer. In experiments investigating the dephosphorylation of phospholamban, phosphorylation was stopped by addition of an inhibitor peptide, amide 5–24 (Peninsula Labs, 500 nM). Dephosphorylation was subsequently quenched by the addition of SDS–PAGE sample buffer. Thiophosphorylation was performed in an identical manner.

Phosphoamino Acid Analysis. Phosphorylated phospholamban was identified in SDS–PAGE gels by autoradiography and excised. The gel pieces were washed 3 times in 50% methanol, and the filter paper support was removed. Acrylamide pieces were dried under N_2 and hydrolyzed in 6 M HCl at 110 °C for 2 h. Amino acids were recovered from solution by drying in a Speed-Vac (Savant) and resuspended in a small volume (10–20 μL) of water. Samples (4 μL) were spotted onto cellulose-based TLC plates along with 25 nmol of phosphoamino acids standards and phenol red. Residues were separated by electrophoresis, 1000 V, 60 min, using a buffer of pyridine/acetic acid/water (1:10:89) and stained with ninhydrin. Labeled amino acids were detected by autoradiography.

Results

Two major species of phospholamban are separated by SDS–polyacrylamide gel electrophoresis, a monomeric form of low molecular weight (P_LL) and an oligomeric form of higher apparent molecular weight (P_LH). The relative proportions of these two components vary with sample pretreatment, temperature and reducing agent concentration being the most important parameters. A second electrophoretic diversity displayed by phospholamban occurs following phosphorylation. In Figure 1, the oligomeric form of phospholamban displays a number of distinct protein species following phosphorylation as was identified by immunoblotting. Prior to the exposure of SR vesicles to ATP and kinase, the oligomeric form of phospholamban existed as a single species.

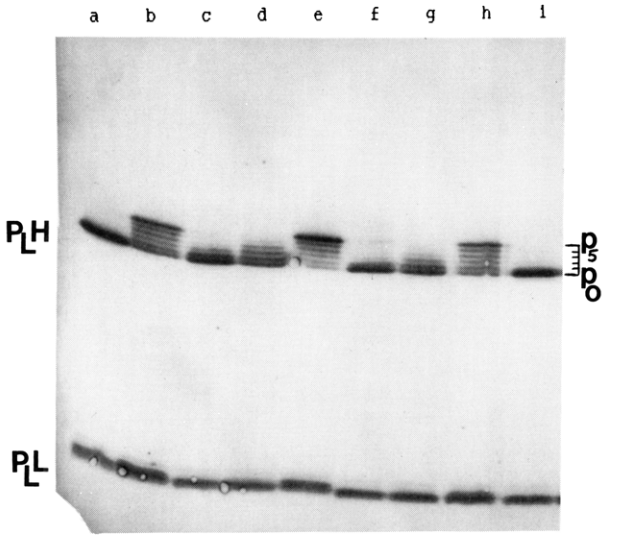

FIGURE 1: Separation of oligomeric phosphorylation species of phospholamban. SR (150 μg) was phosphorylated by cAMP-dependent protein kinase (30 nM) and ATP (0.15 mM) for 0 (a), 0.5 (c), 1 (d), and 3 min (e) in the absence or presence (i, 3 min) of PKI peptide inhibitor (5–24 amide). SR was also phosphorylated for 0.5 (f), 1 (g), and 3 min (b, h) by an endogenous Ca^{2+}-independent protein kinase by the addition of 0.15 mM ATP. Phosphorylation reactions were terminated by the addition of SDS–PAGE sample buffer, and 20 μg of each incubate was resolved on 15% polyacryamide gels. Phospholamban was identified immunologically (details under Experimental Procedures) following transfer of SR proteins to Zeta-probe membranes (Bio-Rad). Oligomeric (P_LH) and monomeric (P_LL) forms of phospholamban are indicated as are the phosphorylation species of the oligomer, P_0–P_5 respectively, a nomenclature which applies to subsequent figures.

Addition of ATP, or ATP plus the catalytic subunit of cAMP-dependent protein kinase, resulted in the generation of a further five phospholamban species of lower electrophoretic mobility. It is likely that these species, as has been suggested previously (Imagawa et al., 1986), are the products of sequential phosphorylation reactions and thus represent nonphosphorylated and mono- to pentaphosphorylated phospholamban (designated P_0, P_1, P_2, P_3, P_4, and P_5 in order of decreasing mobility). The clear resolution of these species was achieved by using standard 15% acrylamide gels made with a low catalyst concentration (0.033% ammonium persulfate/0.025% TEMED). Gels of lower (10%) or higher (20%) acrylamide concentration or with higher catalyst concentration were less able to resolve the phosphorylation species. So too were mini-gels of an identical composition, as would be anticipated.

A parallel retardation of the monomeric species upon phosphorylation has also been described previously (Imagawa et al., 1986; Wegener & Jones, 1984). This was poorly resolved under the conditions described here (slight broadening of the P_LL species in Figure 1, lanes b, e, and h), but alternative conditions, better able to resolve these events, were not considered in this study.

In addition to demonstrating the separation of multiple phosphorylation species of phospholamban, results of Figure 1 show that the purified SR contained a calcium-independent endogenous protein kinase. All six oligomeric phospholamban species are displayed in samples where phosphorylation was catalyzed by the endogenous kinase. The endogenous kinase activity exhibits properties like those of A-kinase. Phosphoamino acid analysis of phospholamban phosphorylated in the presence or absence of exogenous A-kinase showed that phosphoserine was the only phosphoamino acid in the hydrolysate (results not shown). Low concentrations (nanomolar

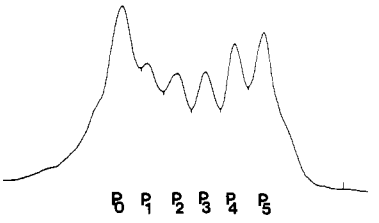

FIGURE 2: Determination of the phosphate content of electrophoretically distinct species. SR vesicles were phosphorylated for 30 s with 0.18 mM ATP (1500 cpm/pmol) and 0.2 μM catalytic subunit of the cAMP-dependent protein kinase. Phospholamban phosphorylation species were resolved on 15% acrylamide gels and detected by immunoblotting (Experimental Procedures). The relative protein concentration of each phosphorylated species was determined from the densitometric tracings and the phosphate content measured by scintillation counting of individual excised species.

range) of the active fragment of A-kinase inhibitor protein (PKI 5–24 amide) prevented the phosphorylation in both cases. In addition, the effects of the two kinases were not additive, and both operated in the absence of calcium and demonstrated a degree of inhibition at calcium ion concentrations of 0.1 mM. As such, the presence of an endogenous A-kinase-like activity will not obscure the description of the mechanism of phospholamban phosphorylation by cAMP-dependent protein kinase.

Phosphorylation by cAMP-Dependent Protein Kinase. The time course of phospholamban phosphorylation by cAMP-dependent protein kinase has been analyzed by immunblotting and autoradiography. The incorporation of phosphate was accompanied by the sequential appearance of phosphorylated species of the protein as was confirmed by autoradiography, and was consistent with the sequential generation of phosphorylation species of phospholamban of ascending phosphate content (data not shown).

The identification of the protein component of phosphorylated species could prove useful for mechanistic studies provided that the phosphate content of each be established unambiguously, and provided the monoclonal antibody be shown to react equally with all species. Assuming the latter to be the case, the mole ratio of phosphate to protein was calculated for each species following a brief exposure to [γ-^{32}P]ATP and A-kinase. Before this task was performed, it was important to verify that the signal intensity of the immunoblot was linearly responsive to protein concentration, since there are instances presented where this relationship appears to have faltered. For example, when present as the sole, or major, species, the signal intensity of P_0 does not reflect its concentration in Figure 1 (lanes a, f, and i) and Figure 4 (lanes 0, 0.25, and 0.5). In these examples, the linear range of the detection system had been exceeded. Such examples cannot and were not used for quantitative analyses. In order to validate the quantitative analyses, immunostaining was shown to respond to protein concentration in a linear fashion by performance of the analysis at two protein loadings. Laser densitometry of resolved pentameric species produced a total integral for each profile which was the precise reflection of the loading relationship of the two samples. The relative concentration of each phosphorylated species was determined by integration of the resolved oligomeric species (a typical example of which is shown in Figure 2) and the phosphate content of each determined following excision from the blot. When the mole ratio of phosphate to protein determined for each species P_0, P_1, P_2, P_3, P_4, and P_5 was plotted against the suggested phosphate stoichiometry of the species, a straight line of unit slope resulted (data not shown). Thus, the phosphorylation status of each species has been confirmed and corresponds to the discrete retardation of species as a function of ascending phosphate content. Demonstration of a clear relationship between phosphate content and electrophoretically resolved species also serves to validate the hypothesis that the monoclonal antibody reacts equally with all species.

A measure of the phosphorylation stoichiometry, at least appropriate to the oligomeric species, can be gained from determination of the relative protein concentrations of these species, according to the equation:

$$\text{stoichiometry} = \frac{b + 2c + 3d + 4e + 5f}{a + b + c + d + e + f} \quad (1)$$

where a–f represent the relative protein concentrations of species P_0–P_5, respectively.

This alternative method of stoichiometry determination may prove to be significant since other approaches depend on a knowledge of the phospholamban concentration in SR. This has proved difficult to measure, and values as disparate as 6% (Le Peuch et al., 1979) and 0.3% (Suzuki et al., 1987) have been described. A method independent of the true phospholamban concentration may prove more reliable in the determination of phosphorylation stoichiometry in SR membranes. It must be stressed, however, that events in the monomeric pool of phospholamban cannot be gauged by this approach, and if different from those of the oligomer would negate the strategy.

Determination of the Kinetic Mechanism on the Basis of the Distribution of Phospholamban Species. A number of kinetic pathways of phosphorylation of the oligomeric phospholamban are possible. The pathway may be cooperative, whereby phosphorylation of one subunit of the pentamer influences the subsequent phosphorylation of another, or noncooperative, in which subunit phosphorlation is independent of the phosphorylation status of the pentamer. The cooperative phosphorylation may be either positive or negative, where phosporylation of a subunit enhances or retards the phosphorylation of another subunit of the same oligomer, respectively. The reaction mechanisms can be distinguished by consideration of the distribution of phospholamban species during the course of phosphorylation. For example, in a reaction showing strong positive cooperativity, the nonphosphorylated and fully phosphorylated species would prevail throughout the course of the reaction. Conversely, in the case of a reaction showing strong negative cooperation, phospholamban with two phosphates would appear only after the nonphosphorylated protein had been entirely converted to a monophosphorylated species.

The relative amounts of each species for a noncooperative reaction (random phosphorylation) can be predicted at any stage by using the binomial equation:

$$(aX + bY)^5 = a^5X^5 + 5a^4bX^4Y + 10a^3b^2X^3Y^2 + 10a^2b^3X^2Y^3 + 5ab^4XY^4 + b^5Y^5 \quad (2)$$

where a and b represent the fraction of nonphosphorylated (X) and phosphorylated (Y) phospholamban subunits, respectively. For example, X^5 represents the nonphosphorylated phospholamban pentamer whereas X^2Y^3 is the triphosphorylated species.

The distribution of phosphorylation species for each of these alternatives in isolation is depicted schematically in Figure 3a for a sample in which the phospholamban has been phosphorylated to a stoichiometry of 2.5 mol/mol (i.e., 50% phosphorylation). Following phosphorylation with 30 nM cAMP-dependent protein kinase and 0.2 mM ATP for various times, the 1-min time point was selected for comparison since it most closely resembled 50% phosphorylation (Figure 3b, 52%

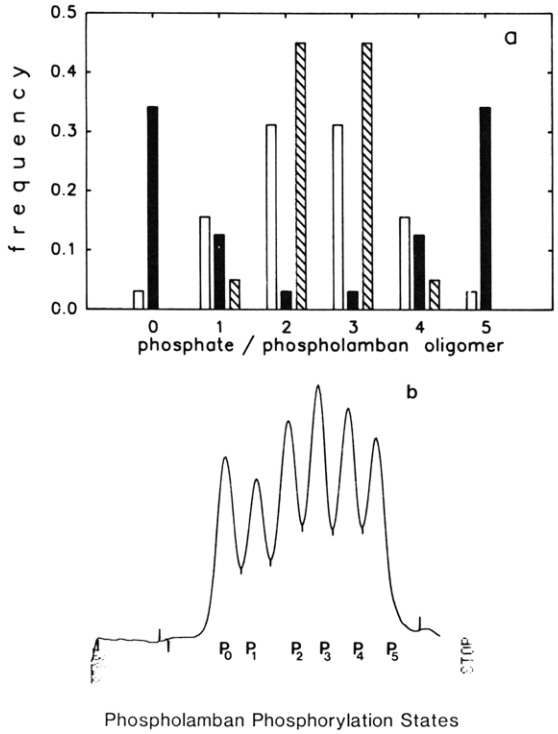

FIGURE 3: Distribution of phospholamban phosphorylation species at approximately 50% phosphorylation. (a) The schematic representation of phospholamban phosphorylation species at 50% maximal phosphorylation assuming a random mechanism (open bar) and mechanisms displaying strict positive (solid bar) and negative (hatched bar) cooperativity. (b) Experimental profile of the relative concentration of phospholamban phosphorylation species at a comparable stage of phosphorylation (52% phosphorylation, calculated according to eq 1; 1-min incubation at 30 °C with 0.2 mM ATP and 30 nM A-kinase).

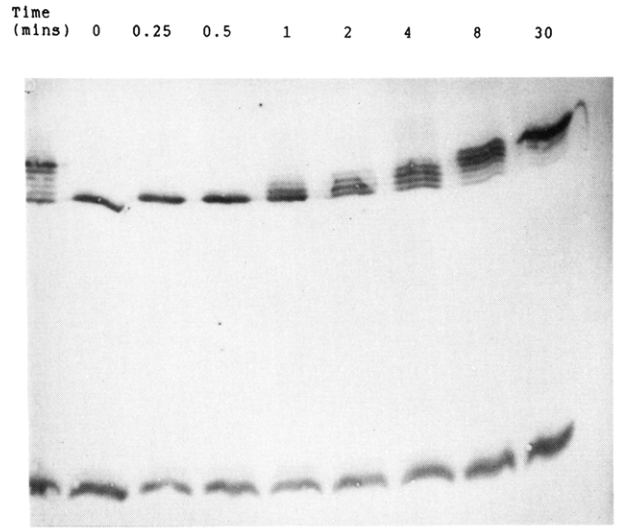

FIGURE 4: Time course of thiophosphorylation of phospholamban by cAMP-dependent protein kinase. SR was thiophosphorylated by 30 nM catalytic subunit of cAMP-dependent protein kinase and 0.2 mM ATPγS for the periods shown. The reaction was performed as described under Experimental Procedures, and phospholamban species were identified following electrotransfer from a 15% polyacrylamide gel. P_0–P_5 nomenclature of the oligomeric species (unmarked) is as denoted in Figure 1.

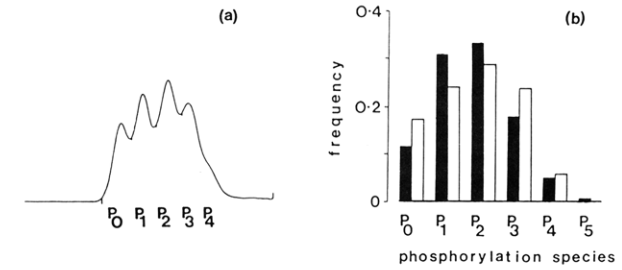

FIGURE 5: Comparison of the species distribution following thiophosphorylation with the theoretical prediction of a random mechanism. The relative protein species distribution of oligomeric phospholamban was analyzed densitometrically (a) from the 4-min time point of Figure 4. A stoichiometry of thiophosphorylation was calculated (1.76 mol/mol) by using eq 1 and the binomial prediction of species distribution at that stoichiometry calculated. Theoretical (solid bars) and experimental (open bars) distributions of phosphorylation species are compared (b).

phosphorylation from application of eq 1). By comparison, it is clear that the A-kinase phosphorylation of phospholamban best fits a random pathway (noncooperative). This is also supported by the sequential generation of phosphorylation species of ascending phosphate content throughout the course of the phosphorylation reaction (data not shown). However, the fit of experimental data to the binomial equation is not perfect since the contribution made by species P_0 significantly exceeds the prediction, as does that of P_5. This could be explained in a number of ways: first, that the result may be artifactual, arising from an inactive component of phospholamban within the SR preparation. Second, the mechanism of phosphorylation may be a complex event involving a mixture of theoretical alternatives, thus deviating from the theoretical ideal of each in isolation. Or lastly, since SR preparations contain endogenous protein phosphatases (Kranias, 1985; Kranias & di Salvo, 1986), the kinetic mechanism of phospholamban phosphorylation might be obscured by the presence of continued dephosphorylation of the phosphorylated phospholamban species. For example, phospholamban dephosphorylation may exhibit cooperative kinetics which are superimposed on the noncooperative phosphorylation, and the sum of the two reactions may then give rise to the pattern of phospholamban species displayed in Figure 3b.

Study of Phosphorylation Kinetics Using ATPγS. The ATP analogue ATPγS has been shown to substitute for ATP as the substrate in many protein kinase reactions (Hoar et al., 1979). The resultant thiophosphorylated protein is usually similar to the corresponding phosphoprotein in biological and molecular properties except that it is largely resistant to the action of phosphatases. The possibility that the use of ATPγS may circumvent the interfering protein phosphatase activity in SR preparations has been investigated. Figure 4 shows that when ATPγS was used instead of ATP, sequential generation of slower moving phosphorylation species was retained, although the rate of the reaction appeared reduced. After 30 min, essentially a single species corresponding to P_5 in mobility is produced. Dephosphorylation of thiophosphorylated phospholamban species was not seen during this time course, as anticipated. These data suggest that the kinetic mechanism of thiophosphorylation is similar to that of phospholamban phosphorylation and that the thiophosphorylation reaction is resistant to the activity of endogeneous protein phosphatases. In addition, the almost complete conversion of P_0 species to P_5 species has excluded the suggestion that a portion of phospholamban in SR is resistant or inaccessible to the action of the protein kinase.

In the absence of a phosphatase reaction, patterns of the phospholamban species distribution during the course of the A-kinase-catalyzed reaction are expected to be dictated by the kinetic mechanism of the phosphorylation reaction alone. Figure 5a shows the densitometric tracing of the oligomeric species of a sample from the preceding figure (Figure 4, time

4 min, 35% phosphorylation). This has been integrated and the results (Figure 5b) substituted into the formula of eq 1 to obtain a stoichiometry of phosphorylation of 1.76 mol/mol. On the basis of this stoichiometry, the distribution of phospholamban species for a completely random phosphorylation reaction can be predicted by using the binomial equation (eq 2). Figure 5b shows that the experimentally derived distribution correlates closely with that of the prediction. The results indicate that thiophosphorylation of phospholamban by cAMP-dependent protein kinase follows a noncooperative kinetic pathway.

Kinetic Mechanism of Dephosphorylation. The results of thiophosphorylation of phospholamban suggest strongly that phosphorylation of phospholamban catalyzed by A-kinase is a noncooperative reaction. Assuming therefore that phosphorylation be a completely random event, the deviation of the phosphorylated species distribution (data in Figure 3b) from that of the ideal would indicate that the dephosphorylation reaction must show positive cooperativity. To determine the kinetic mechanism of phospholamban dephosphorylation, a sample of SR was fully phosphorylated using a high concentration of cAMP-dependent protein kinase. The kinase activity was then blocked by addition of an excess of the kinase inhibitory peptide (PKI 5–24 amide) and the phosphatase reaction followed. Figure 6 shows the densitometric analysis of oligomeric species during the dephosphorylation process. Although close to complete dephosphorylation took more than 60 min, fully dephosphorylated phospholamban, P_0, began to accumulate 30 s after addition of the kinase inhibitor. Little or no change in the concentration of phosphorylation species of intermediate phosphate content (P_1–P_4) was observed throughout the process of dephosphorylation. The results demonstrate that the dephosphorylation reaction follows a strict positively cooperative kinetic mechanism. This finding further supports the conclusion drawn from thiophosphorylation studies that phospholamban phosphorylation by A-kinase follows a noncooperative reaction pathway, since it offers a compelling explanation for the discrepancy in P_0 concentration between these two reactions.

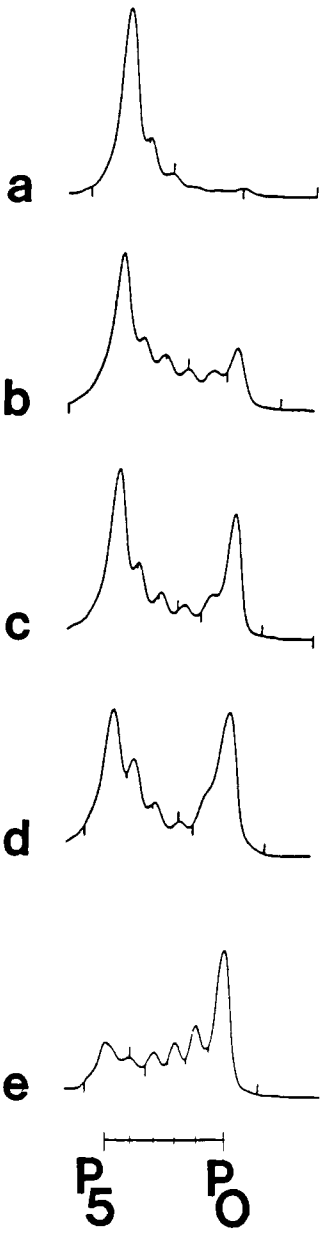

FIGURE 6: Dephosphorylation of A-kinase phosphorylated phospholamban. SR was phosphorylated by 127 nM catalytic subunit of the cAMP-dependent protein kinase and 0.15 mM ATP (790 cpm/pmol) for 3 min. The kinase activity was terminated by addition of 500 nM PKI (5–24 amide) and dephosphorylation followed over 60 min. Phospholamban species were identified as described in previous figures, and the densitometric analysis of the oligomeric species is presented (a) immediately preceding addition of PKI; and 0.5 (b), 1 (c), 3 (d), and 20 (e) min thereafter.

Discussion

Identification of phospholamban in preparations of sarcoplasmic reticulum by immunological means has enabled description of a number of electrophoretically distinct species following protein phosphorylation. A total of six oligomeric species were identified when phospholamban was phosphorylated by cAMP-dependent protein kinase or by an endogenous kinase. This is entirely consistent with the pentameric model of phospholamban. The degree of retardation of these species correlated precisely with the increasing phosphate content of the oligomer, 0–5 mol/mol, thus endorsing P_0–P_5. Assessment of the protein composition of each phosphorylated component facilitates the performance of two important tasks: (i) the calculation of stoichiometry independent of a knowledge of phospholamban concentration; (ii) description of the mechanism of phosphorylation and dephosphorylation. A-kinase phosphorylation follows a noncooperative route whereas dephosphorylation is strongly cooperative.

The generation of numerous oligomeric species of phospholamban following phosphorylation was previously reported by Imagawa et al. (1986). In that study, five phosphorylated species of progressively increasing apparent molecular weight were generated following phosphorylation of phospholamban by A-kinase. Comparison of the phosphopeptide composition of individual species was used to describe the subunit composition of oligomeric phospholamban. Multiples of a single unit (monomer) were observed, and the data were taken as evidence in support of the pentameric arrangement of phosholamban. Potential shortcomings of this approach in the description of the subunit arrangement of a protein cannot be excluded. For example, an inaccurate description of the polymeric composition of phospholamban would arise as a consequence of either comigration of two distinct phosphorylation species or electrophoretic discrimination between the theoretical permutations of a single phosphorylated species (di- and triphosphorylated species in the case of a pentamer).

Aspects of the present study serve as an extension of the former in that description of both the protein and phosphate components of individual species has enabled confirmation of assumptions made by Imagawa et al. (1986) concerning the phosphorylation status of each species. We have been able to demonstrate that the species designated P_0–P_5 inclusive in

this study contain 0–5 mol of phosphate/mol of protein, as was previously assumed. The data also afford an assurance that the monoclonal antibody reacts equally with all species and that densitometric quantification of protein concentration from data of the quality presented is accurate and reliable, a prerequisite for the description of the mechanism of phosphorylation.

It is possible to conclude from these studies that the generation of five phosphorylation species of phospholamban is consistent with a pentameric arrangement of the protein, since a single phosphorylation site has been described, by others, for each monomer using A-kinase (Simmerman et al., 1986).

The description of the relative protein concentration of each species can be used as an alternative means of determining phosphorylation stoichiometry. This method may prove superior to that of radiolabeled phosphate incorporation since it does not require an accurate knowledge of the phospholamban concentration in an SR preparation. The validity of this approach is dependent on the identity of oligomeric and monomeric species with respect to kinase action. Such an assumption may be justified since it is currently unclear whether monomeric forms of phospholamban persist in the membrane or arise as a consequence of electrophoresis. Studies to address these issues are currently in progress.

Perhaps the most important advance facilitated by measurement of the protein component of individual phosphorylation species is the ability to address the mechanism of phosphorylation and dephosphorylation. A similar approach has been used to describe the random phosphorylation of the two light chains in smooth muscle myosin (Trybus & Lowey, 1985). Separation of mono- and diphosphorylated light chains by nondenaturing gel electrophoresis allowed detection of the relative concentration of phosphorylated species. These data were compared to theoretical exponentials and cooperative mechanisms of phosphorylation excluded. Description of the mechanism of phospholamban phosphorylation is increased in difficulty by virtue of the pentameric structure and presence of a large number of other SR proteins. Nevertheless, by eliminating the reverse reaction (dephosphorylation, ATPγS), the distribution of individual phosphorylation species was shown to closely resemble that predicted by the binomial equation (eq 2) characteristic of a random mechanism. Thiophosphorylation of phospholamban by cAMP-dependent protein kinase follows a random mechanism; phosphorylation deviates from this relationship in that the nonphosphorylated species is present in unexpected abundance. The possibility that this deviation was due to a positively cooperative dephosphorylation mechanism was confirmed. Dephosphorylation was shown to be strictly cooperative, with hydrolysis of the first phosphate greatly enhancing the subsequent removal of remaining phosphates from a single pentamer. Thus, the slight deviation in the distribution of phosphorylated protein species was produced by superimposition of a random-order phosphorylation reaction and positive cooperation in the removal of the phosphate moiety from a serine, which has been shown by others to be serine-16 (Simmerman et al., 1986).

The functional significance of these findings is not apparent at this juncture. By definition, however, phosphorylation via a random pathway would result in the coordinated phosphorylation of phospholamban at the population level, whereas the cooperative nature of dephosphorylation would result in a split population of fully phosphorylated and fully dephosphorylated protein. The initiation and termination of ATPase stimulation would share this disparity, although the consequence of such action is unknown.

The technique of identification of individual phosphorylation species described here offers the potential for studying the fine details of phospholamban control of the ATPase. Recently, Kasinathan et al. (1988) demonstrated a correlation between ATPase activity and the degree of phospholamban phosphorylation. This study was performed at a population level, and we are in the process of applying the present techniques to address such relationships at the molecular level.

Acknowledgments

We thank Dr. M. P. Walsh for assistance and access to the LKB Ultrascan.

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