Vol. 105, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1982 Pages 152-156

PHOSPHOLAMBAN: DISSOCIATION OF THE 22,000 MOLECULAR WEIGHT PROTEIN OF CARDIAC SARCOPLASMIC RETICULUM INTO 11,000 AND 5,500 MOLECULAR WEIGHT FORMS

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Received January 21, 1982

Phospholamban, originally identified as a 22,000 dalton protein of cardiac sarcoplasmic reticulum where it is involved in the regulation of calcium transport, can be dissociated into 11,000 and 5,500 dalton forms as determined by polyacrylamide gel electrophoresis. The 22,000 dalton form is obtained by solubilizing microsomes in sodium dodecyl sulphate at 37°. Immersing sodium dodecyl sulphate-denatured microsomes in boiling H_2O for one min or five min results in a shift to the 11,000 and 5,500 dalton forms, the latter predominating and presumably representing the monomeric form of phospholamban. These findings can reconcile some of the discrepant findings on phosphoproteins in cardiac sarcoplasmic reticulum reported by different laboratories.

Calcium transport in sarcoplasmic reticulum vesicles has been shown to be regulated by cyclic AMP- and calmodulin-dependent protein kinases (1-7). These protein kinases catalyze the phosphorylation of a membrane-bound protein called phosphlamban whose molecular weight remains uncertain. Phospholamban was originally identified as a 22,000 molecular weight protein by sodium dodecyl sulphate[1]-polyacrylamide electrophoresis (8) but recently was reported to be dissociated into monomers of 11,000 daltons by treatment with Triton X-100 (9). We now present evidence for the further dissociation of phospholamban into an apparent monomeric form of 5,500 daltons.

MATERIALS AND METHODS

Microsomes consisting mainly of fragmented sarcoplasmic reticulum were obtained from canine ventricle by previously described methods (10). Briefly, these methods consist of tissue homgenization, two centrifugations at 10,000 x g for 30 min and two centrifugations at 95,000 x g for 75 min, including one wash in 0.6 M KCl. Protein concentrations were determined by

[1] Sodium dodecyl sulphate, SDS

0006-291X/82/050152-05$01.00/0

biuret method with bovine serum albumin as the protein standard. Calmodulin was partially purified through the DEAE-cellulose chromatography step of the method of Sharma and Wang (11) modified slightly (10).

To phosphorylate microsomes, microsomes (1.5 mg/ml) were incubated for one min at 25° C in 50 mM histidine-HCl, pH 6.8, 120 mM KCl, 5 mM NaN_3, 1 mM ouabain, 25 mM NaF, 5 mM $MgCl_2$, a Ca-EGTA buffer (10) to result in a final Ca^{2+} concentration of 0.66 μM, 6 μM calmodulin, and 20 μM ATP containing [γ-^{32}P]ATP at a specific radioactivity of 4×10^9 cpm/μmol. The volume was 100 μl. Reactions were stopped by adding 2 ml of ice-cold 10% trichloroacetic acid/1 mM NaH_2PO_4. Pellets obtained upon centrifugation were washed once with water and solubilized in SDS (8). Solubilized microsomes were heat-treated as described in the legend to the figure.

Samples were electrophoresed on slab gels prepared by the methods of Laemmli and Favre (12) modified in that a 7 to 20% polyacrylamide gradient was used. Details on electrophoretic procedures were described previously (10). Slab gels were fixed for 4 h in 7% acetic acid and placed in plastic bags. They were then placed on Kodak NS-5T x-ray film in light-proof x-ray exposure holders and left for 24 h after which time the film was developed.

RESULTS AND DISCUSSION

Autoradioography of cardiac microsomes phosphorylated in the presence of [γ-^{32}P]ATP and calmodulin by endogenous calmodulin-dependent protein kinase contain ^{32}P label localized almost exclusively to a single protein corresponding to a molecular weight of 22,000 (Fig. 1, sample 1, band d,). The SDS-denatured microsomes were warmed to 37° C for 10 min prior to being applied to the gels, as described previously (10). Similar results were obtained when the incubation time was two h or when the protease inhibitor phenylmethylsulphonyl fluoride was included in the SDS solubilization mixture. Trace amounts of ^{32}P label were seen at locations on the gel marked a to c, e, and f in Fig. 1, sample 1. The possible identity of these phosphoproteins was discussed previously (10).

Treatment of the SDS solubilized microsomes in boiling water for one min resulted in the complete dissociation of the 22,000 molecular weight protein into proteins of 11,000 (band e) and 5,500 (band f) daltons, the latter predominating (Fig. 1, sample 2). A heat-treatment period of 5 or 10 min resulted in a slight further dissociation of the 11,000 dalton protein into the 5,500 dalton form (Fig. 1, samples 3 and 4). The mobilities of proteins a through c remained unchanged. These results suggest that the monomeric form of phospholamban is a polypeptide of 5,500 daltons and that the 22,000 dalton protein previously identified represents a tetramer.

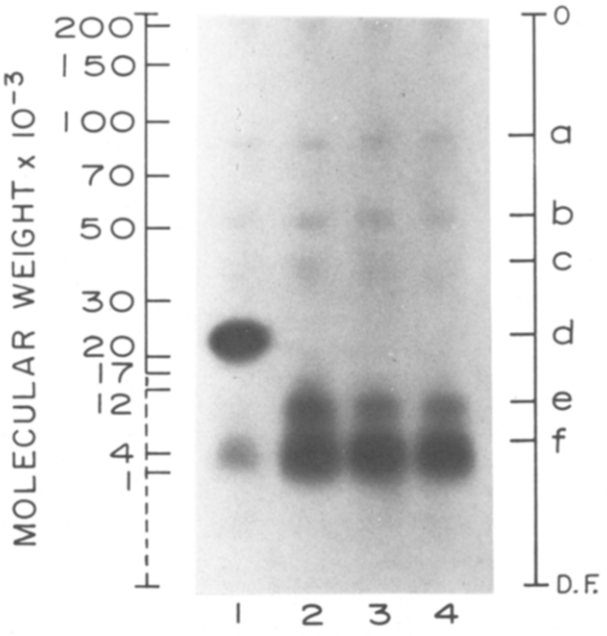

Fig. 1. Autoradiogram of sodium dodecyl sulphate-polyacrylamide electrophoretic gel of ^{32}P-labelled canine cardiac microsomes. See text for phosphorylation conditions. Microsomes were solubilized in sodium dodecyl sulphate and incubated at 37° for 10 min (sample 1), or placed in a boiling water bath for 1 min (sample 2), 5 min (sample 3), or 10 min (sample 4). Each sample consists of 150 µg microsomal protein. Letters refer to ^{32}P-labelled substrates corresponding to the following approximate molecular weights: (a) 93,000; (b) 55,000; (c) 40,000; (d) 22,000; (e) 11,000; and (f) 5,500. The origin (0) and dye front (D.F.) are indicated. An apparently linear relationship between the log of the molecular weight and the protein mobility exists in the molecular weight range between 200,000 and 17,200, indicated by a solid line on the calibration scale. In the nonlinear range indicated by a broken line, molecular weights of the following standard proteins are shown: cytochrome c (12,400), glucagon (3,600), bacitracin (1,400).

The identification of a 5,500 molecular weight form of phospholamban can account for the seemingly discrepant reports in the literature concerning the presence of 22,000, 11,000 and 6,000 or 7,000 dalton phosphoproteins in mammalian myocardium. The distribution of these forms of phospholamban depends largely but not entirely on the conditions used to solubilize the microsomes. Thus vigorious heat treatment as by boiling yields largely the 5,500 molecular weight form while mild treatment at 37° C for 10 min yields almost entirely the 22,000 molecular weight form (Fig. 1). Other factors besides heat treatment of the SDS solubilized microsomes appear to be operative. Bidlack and Shamoo (13), who treated SDS-solubilized microsomes at

37° C for 1 h, found almost all ^{32}P labelling associated with the 22,000 molecular weight form when microsomes were phosphorylated for only one min but, in contrast to our data (10), found equal or greater phosphorylation of a 6,000 dalton form when the phosphorylation period was increased to 30 min. Jones et al. (14) solubilized microsomes phosphorylated for one min in SDS at room temperature, yet found an approximately equal distribution of 22,000 and 7,000 molecular weight phosphorproteins. Le Peuch et al. (9) concluded that the monomeric species of phospholamban is 11,000 daltons although, they pointed out, their amino acid analysis of this protein was consistent with a minimal molecular weight of 5,500. They found that when the 22,000 dalton species was eluted from the gel into SDS solution containing 1% Triton X-100 and re-electrophoresed, the protein moved with a mobility indicating the molecular weight was 11,000.

Onorato and Rudolph (15) recently studied phosphorylation of proteins in isolated rat myocardial cells and found an increase in phosphorylation by isoproterenol of a 12,000 molecular weight protein which was detected in samples that had been boiled for 5 min. These investigators contend that the 12,000 molecular weight protein is not phospholamban because Triton X-100 was not utilized in the gel system. Since a boiling step was utilized, the 12,000 dalton protein appears to be phospholamban in light of the data presented in this communication. The 5,500 molecular weight form appears not to be resolved by their gels and may be obscured in the mass of ^{32}P shown at the bottom of the autoradiograms.

The identification of the monomeric unit of phospholamban as a 5,500 molecular weight protein should facilitate studies aimed at its chemical characterization and elucidation of the mechanism of its interaction with the calcium pump of cardiac sarcoplasmic reticulum. This protein may play a key role in regulating calcium transport in the sarcoplasmic reticulum during myocardial relaxation and in mediating the relaxation promoting effects of catecholamines on the intact heart (16).

ACKNOWLEDGEMENTS - This work was supported by a Grant-In-Aid from the New York Heart Association and grant HL 15764 from the United States Public Health Services, NIH.

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