SARCOPLASMIC RETICULUM ATPase ON A SOLID SUPPORT

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A metal-sensitive ouabain-inhibited ATPase activity of rabbit heart sarcoplasmic reticulum has been studied. From the membrane preparation a solubilized ATPase has been prepared by treatment with digitonin. The active fraction has an approximate molecular-weight of 100,000 - 150,000, estimated upon the basis of sedimentation and gel filtration characteristics. The solubilized magnesium-stimulated enzyme is only slightly and erratically sensitive to ouabain; insensitive to $Na^+ + K^+$. The enzyme was linked to a reactive cellulose matrix and the activity again characterized. ATP-hydrolysis was catalyzed by the matrix-supported preparation. This activity was greatly inhibited by the presence of 10^{-4} M ouabain.

If those characteristics which are taken as criteria (Skou, 1965), for recognition of membrane-derived transport ATPase (metal and cardiac glycoside response) are related to the structure, not only of the enzyme itself but of the membrane with which it is associated, then solubilization from the membrane should alter these properties. Further, if the enzyme characteristics in question are a function of the protein conformation resulting from enzyme association with the biological membrane, then it may be possible by linkage of the enzyme to a polymeric support to regain the original characteristics. Using a preparation from sarcoplasmic reticulum of rabbit heart (Schwartz, 1962; Brown, 1966a; 1966b) we have tested the thesis that the transport-associated characteristics of membrane ATPase vary in response to such structural interrelationships.

Methods.

Frozen rabbit hearts were macerated in ten volumes of cold Tris buffer, 0.1 M, pH 7.2 with 0.25 M sucrose and the slurry was centrifuged at 600 x g for 20 minutes. Pellets were rejected and the supernatant was dialyzed for 24 hours against the same Tris sucrose buffer with 5 mM

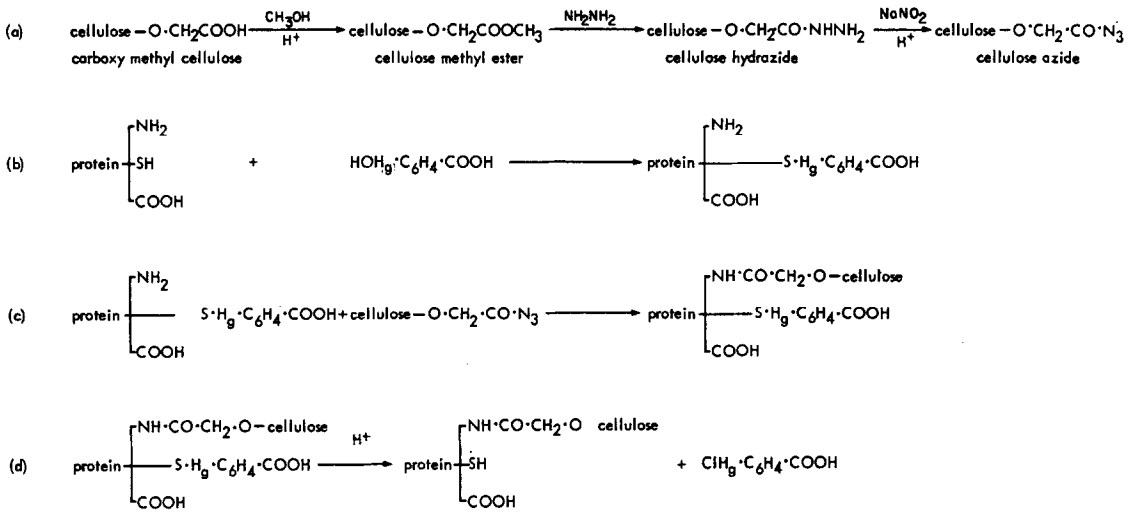

Fig. 1. (a) Cellulose azide, to serve as the reactive matrix, is prepared as shown. (b) Sulfhydryl groups of the enzyme are protected by reaction with hydroxymercuribenzoate. This presumably serves to prevent binding to residual carboxy methyl groups of the matrix. (c) The enzyme mercuribenzoate complex is reacted with the cellulose azide and (d) the mercuribenzoate hydrolyzed in the presence of HCl.

disodium ethylendiamine tetraacetic acid and Tris-saturated Amberlite IRC-50 resin. The dialyzate was again centrifuged 10,000 x g for 30 minutes and 20,000 x g for 30 minutes. The supernatant was then centrifuged in 10 ml tubes, 80,000 x g for 30 minutes. Each time the pellets were discarded. Finally the supernatant was centrifuged 100,000 x g for 70 minutes. These pellets were resuspended in 2 ml Tris-sucrose buffer pH 7.2. This was taken as the sarcoplasmic-reticulum fraction.

'Solubilized' ATPase was obtained by treating the membrane fraction with digitonin. A 2% digitonin suspension, prepared just before use, was added to the membrane preparation to a final concentration of 0.5%. This was stirred for 30 minutes and left undisturbed for another 25 minutes. Finally it was homogenized in a teflon-pestle homogenizer for 1 to 2 minutes. It was again centrifuged at 100,000 x g for 70 minutes. The supernatant was taken as solubilized ATPase. This preparation was characterized by sedimentation, gel filtration, pH activity optimum, and metal- cardiac-glycoside-response. Gel filtration was accomplished with a Sephadex 2.5 cm x 45 cm column using 8 grams of dextran G-150 to produce a column

39 cm in length (for other dextran grades a weight was chosen to produce column beds of the same height.) Collections were made within a refrigerated fraction collector and the peaks identified by the Lowry protein technique and by phosphate assay.

Solubilized enzyme was chemically linked to cellulose azide (Mitz and Summaria, 1961). Four ml of solubilized enzyme were stirred with 2 mg of p-hydroxy mercuribenzoate for 1/2 hour. Then, 40 mg of cellulose azide was added to the mixture and the whole preparation stirred again for $1\frac{1}{2}$ to 2 hours and kept overnight at 4-6°. The reactions are described by Figure 1. After centrifugation, pellets were collected and washed twice in 10^{-6} M HCl. The final washed pellet was resuspended in 1.5 ml deionized water. Solubilized and matrix-supported enzymes were used immediately after preparation.

ATPase activity was measured in the reaction mixture containing 0.1 ml enzyme, 0.8 ml substrate [Tris sucrose buffer, pH 7.2 with disodium ATP or Tris ATP (0.3 mg/ml of reaction mixture) together with 0.001 M NaCl, 0.002 M KCl, 0.001 M $MgCl_2$], 0.1 ml water (or 10^{-4} M ouabain). Reaction mixtures were incubated at 42°C for 60 minutes. Then 0.1 ml cold trichloroacetic acid (50%) was added and the mixtures were centrifuged at 600 x g for 6 minutes. The supernatant was assayed for inorganic phosphate (Fiske and SubbaRow, 1925). Protein was determined by the phenol method (Lowry, *et al.*, 1951).

Results and Discussion.

The solubilized enzyme has, upon the basis of sedimentation and gel filtration rates, a molecular weight between 100,000 and 150,000. The active fraction does not sediment when centrifuged for 2 hours at 100,000 x g in a Beckman #50 rotor but does sediment completely after 3 hours under the same conditions. These preparations chromatograph on Sephadex G-150 but are totally excluded on G-100 and smaller-pored gels. Chromatography on G-150 produces several active peaks. Digitonin solubilization appears to fragment the membrane into a cluster of particles, within this molecular-weight range, which bear the active protein. The ATPase of the membrane fraction has a single activity maximum at pH 8.1, while solubilized and matrix-supported preparations have activity maxima at pH 7.2. This result may be interpreted in terms of purification if it is assumed that contaminating protein is present in the membrane fraction. This thesis is not entirely satisfying however in view of the fact that ouabain sensitivity of the membrane fraction was constant for the range pH 6.8 - 9.2 and the solubilization and support linkage steps accomplish only minor purifications. (When

activity is stated in terms of phosphate evolved per time as a function of protein, the solubilized preparation represented an increase of 55% over the membrane fraction.)

In preparations of the membrane fraction containing 0.2% sodium deoxycholate, ouabain consistently inhibited the ATPase activity (Brown, 1966b). Solubilized preparations however were quite variable in their response to ouabain, though the effects were always very small, with slight stimulation predominating (Table I).

TABLE I

The effect of ouabain upon ATPase activity.

Preparation	μmoles Pi/mg Protein/min			Percent change in activity
	Control	10^{-4} M ouabain	Variation*	
A. solubilized				
427	2.76	3.20	±0.10	+ 15.9
512	3.50	3.05	0.10	-12.0
524	6.13	6.16	0.10	+ 0.4
526	4.97	5.17	0.10	+ 4.0
614	1.92	1.92	0.10	0.0
107	1.49	1.42	0.20	- 4.5
712	3.44	3.67	0.10	+ 6.1
720	5.00	5.15	0.15	+ 3.0
B. matrix-supported				
R712	1.42	0.60	0.20	-57.7
R715	6.40	1.87	0.30	-70.8
R719	1.99	0.30	0.10	-84.4
R725	1.58	0.83	0.00	-47.5

* Results given as average and variation of six determinations.

Matrix-supported enzyme preparations were inhibited by 10^{-4} M (Table I). Thus responses to ouabain of the three types of preparations; membrane, solubilized, and matrix-supported differed from each other. The effects of metals similarly related to the type of preparation. In experiments, the concentration of one of the three metals was varied while

concentrations of the other two were held constant. Each of the three preparations was stimulated by magnesium with an optimum at 0.001 M (as a chloride). The membrane fraction was stimulated optimally when the endogenous NaCl and KCl levels were 0.001 M and 0.002 M, respectively. The solubilized and matrix-supported preparations were essentially unaffected by these metals though high levels (0.1 M) were inhibitory.

These data may be interpreted theoretically in terms of protein conformation. We suggest that the response to ouabain and to Na^+ and K^+ is a function of the enzymes 3-dimensional configuration. Thus, the *in vivo*, membrane-imposed conformation results in sensitivity in the ouabain and to the metal ions. This is lost with the conformation change that accompanies solubilization. Linkage to an insoluble matrix again imposes upon the enzyme a configuration the result of which is the response to ouabain. Failure to find a $Na^+ + K^+$ sensitivity in the matrix supported preparation is an indication that we have not completely restored the enzyme to its *in vivo* conformation even though the ouabain site has been restored. This thesis is of utility in the design of the continuing investigation.

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