BRIEF COMMUNICATION

ATP SYNTHESIS BY Ca^{2+} + Mg^{2+}-ATPASE IN DETERGENT SOLUTION AT CONSTANT Ca^{2+} LEVELS

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ABSTRACT ATP has been synthesized by the purified Ca^{2+} + Mg^{2+}-dependent ATPase from sarcoplasmic reticulum (SR) solubilized in nonionic detergent dodecyloctaoxyethylenglycolmonoether in a solution containing inorganic phosphate and glycerol by changing pH upon addition of ADP. The Ca^{2+} concentration is kept constant during the experiment. Optimum synthesis is found at $CaCl_2$ = 0.6 mM and the $\Delta pH = 2.9 \pm 0.2$. The enzyme has been digested by trypsin for 1 and 20 min, and it is found that synthesis of ATP is correlated with the Ca^{2+}-uptake into SR. The data indicate that the enzyme alone is responsible for active transport of Ca^{2+} in SR. The driving force for the ATP synthesis of the process may be due to various ion-protein interactions. H^+ cannot substitute for Ca^{2+} in the synthesis of ATP but acts probably through a modification of the Ca^{2+} binding sites. The data give support that the integrity of the enzyme molecule between its hydrolytic site and the Ca^{2+}-binding sites is essential for the overall Ca^{2+} transport.

INTRODUCTION

The mechanism of energy transfer between the ATP hydrolytic site and the Ca^{2+} binding within the Ca^{2+} + Mg^{2+}-ATPase[1] is the subject of extensive study by our laboratory and others (Shamoo and Goldstein [1] and Blumenthal and Shamoo [2]). Knowles and Racker (3) proposed that energy derived from ion-protein interactions can drive the synthesis of ATP in the reversal of the transport process. This was concluded from experiments synthesizing ATP with leaky SR vesicles by changing the Ca^{2+} concentration of the solution. More recently de Meis and Tume (4) repeated the above synthesis and found that ATP can be synthesized at constant Ca^{2+} concentration by forming a pH gradient across the membrane of sealed SR vesicles.

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[1] *Abbreviations used in this paper:* Ca^{2+} + Mg^{2+}-ATPase, Ca^{2+} + Mg^{2+}-dependent adenosine triphosphatase; $C_{12}E_8$, dodecyloctaoxyethylenglycolmonoether; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesylfonic acid; P_i, inorganic phosphate; SR, sarcoplasmic reticulum; TLC, thin-layer chromatography.

If the protein alone is sufficient for transport, then it should be possible to synthesize ATP using solubilized Ca^{2+} + Mg^{2+}-ATPase with the sudden change of pH in the reaction solution. le Maire et al. (5) have shown that the Ca^{2+} + Mg^{2+}-ATPase in true solution retains the enzyme activity. This report presents evidence that ATP is synthesized with Ca^{2+} + Mg^{2+}-ATPase that is solubilized by the nonionic detergent $C_{12}E_8$. $C_{12}E_8$ has been shown by Dean and Tanford (6) to solubilize Ca^{2+} + Mg^{2+}-ATPase with full maintenance of enzymatic activity. Furthermore, this report gives further evidence that the integrity of the energy transduction step of Ca^{2+} + Mg^{2+}-ATPase molecule between the Ca^{2+} binding sites and ATP hydrolytic site is necessary for the capability of the enzyme to synthesize ATP molecule.

MATERIALS AND METHODS

Materials

The detergent $C_{12}E_8$ was the nominally pure compound prepared by Nikko Chemicals Co., Tokyo, Japan. Octyl glucoside was synthesized; see Zimniak and Racker (7) for further details. The following chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.): ADP grade IX grom equine muscle, hexokinase type C-120 from yeast, trypsin, and soybean trypsin inhibitor. ^{32}Pi and [γ-^{32}Pi] ATP was obtained from Amersham Corp. (Arlington Heights, Ill.). Nucleotides were separated on the Baker-flex sheet cellulose polyethyleneimine from J. T. Baker Chemical Co. (Phillipsburg, N.J.).

Preparations

SR vesicles were prepared from rabbit skeletal muscle according to MacLennan (8) (washed R_i of preparation A). Purified Ca^{2+}-Mg^{2+}-ATPase was obtained by octyl glucoside extraction as referred to by Zimniak and Racker (7). Final concentration of octyl glucoside in the protein solution was 20 mM.

Controlled tryptic digest of SR was performed by essentially the same method as reported by Ikemoto et al. (9). The trypsin-to-protein ratio was 2:1 (milligram:milligram) and the ratio of trypsin inhibitor to trypsin used was 4:1 (milligram:milligram). Digestion was carried out at 22°C for 1 and 20 min to give, respectively, the compositions TD1 and TD2. The tryptically digested SR was washed and extrinsic proteins removed as described by Scott and Shamoo (10). The protein preparations were subjected to polyacrylamide gel electrophoresis using the method of Weber and Osborn (11). The destained gels were scanned at 550 nm in a Gilford 240 spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio). The areas under the peaks corresponding to each of the fragments were determined and normalized such that the sum of areas was equal to 1.0. The gels run on the preparations SR, Ca^{2+} + Mg^{2+} ATPase, and tryptically digested compositions TD1 and TD2 are shown in Fig. 1. Compositions are given in the figure legend.

Ca^{2+}-dependent ATPase activity was determined isotopically using [γ-^{32}P] ATP. The assay medium was 100 mM KCl, 5 mM $MgCl_2$, 50 mM Hepes, KOH pH = 7.0, 100 μM $CaCl_2$. Liberated radioactive P_i was determined by extraction with ammonium-molybdate into butylacetate (Sanui [12]). The activities were in micromoles P_i per milligram per minute around 2.0 for freshly prepared SR, and 4 for Ca^{2+}-Mg^{2+}-ATPase.

The fragmented compositions were made from 10-wk-old SR preparation. After this time the activities were 0.2 for SR, TD1, and TD2. Protein concentrations were determined by absorbance at 280 and 260 nm on a Gilford 240 spectrophotometer (Gilford Instrument Laboratories Inc.).

Reaction Conditions

The reaction between ADP and P_i with the soluble Ca^{2+} + Mg^{2+}-ATPase was carried out in the following way: approximately 1 mg of the enzyme preparation was first dissolved in a 160-μl mixture of 2% $C_{12}E_8$, 50% vol/vol glycerol, and 25 mM maleic acid, pH = 5.1. Salt solution, distilled deionized water, and in some cases 80 units of hexokinase was added to give a final volume of 400 μl. The salt

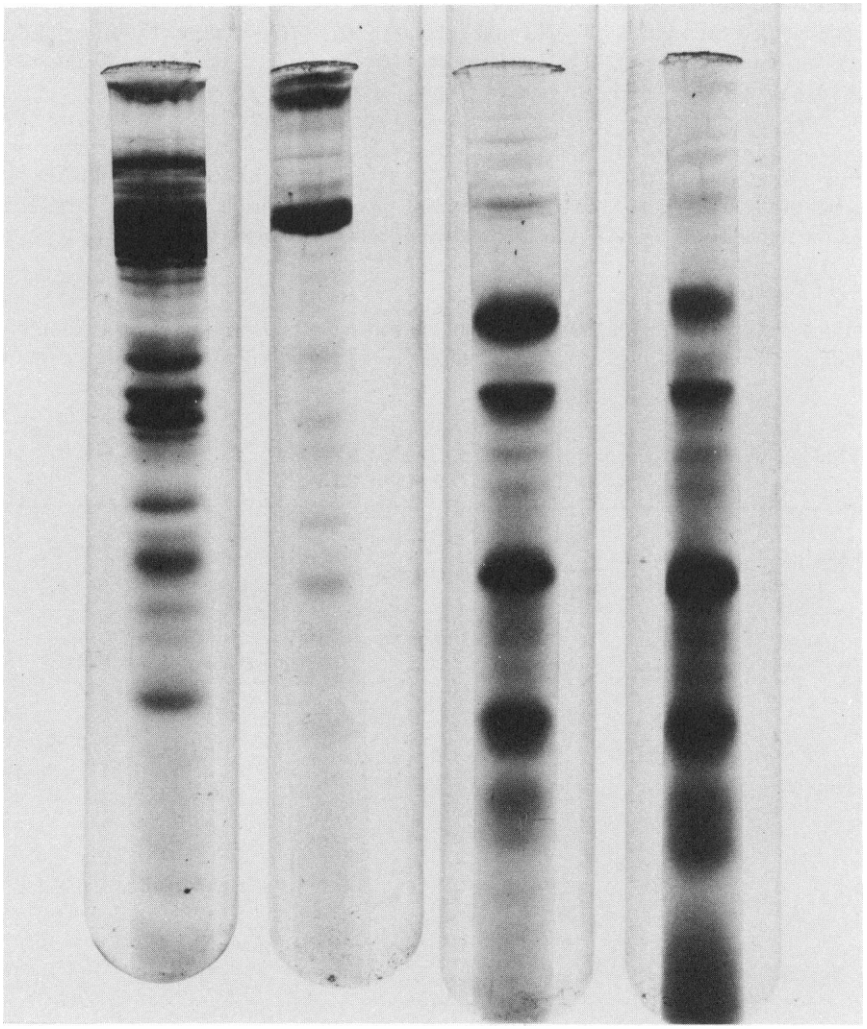

FIGURE 1 7½% acrylamide gel electrophoresis of SR (gel 1), Ca^{2+} + Mg^{2+}-ATPase (gel 2), and SR exposed to trypsin for 1 and 20 min (gels 3 and 4, respectively). Scanning spectroscopy of gels at 550 nm yields weight percent of fragment A in gels 3 and 4 equal to 30 and 10.

concentrations in the final mixture were 10 mM $MgCl_2$, 4 mM $^{32}P_i$(>10 8 cpm/μmol), and 0.6, 0.3, 0.1, or 0 mM $CaCl_2$.

EGTA was added to give 1 mM in the final solution when $CaCl_2$ was not present. The protein:detergent:glycerol ratio in this mixture should give a stable, active, soluble enzyme (6). The enzyme was usually allowed to phosphorylate for 0.80 min in this mixture. Then 100 μl of a solution containing ADP, $CaCl_2$, KOH, and glucose in the presence of hexokinase was added. We added ADP to give a final concentration equal to 4 mM, glucose equal to 5 mM, $CaCl_2$ to maintain the concentration of the starting solution, and KOH to give the desired pH.

The reaction temperature was 37°C and a shaking water bath was used to maintain the temperature. Solutions were equilibrated in this water bath before use. The test tubes were taken from the water baths for short periods of vortexing after each addition.

The reaction was terminated after 0.85 min with 80 μl ice-cold 25% perchloric acid to liberate tightly bound nucleotides, as described by Scott and Shamoo (10). After incubation on ice for 5 min, the precipitate was obtained by centrifugation in a table top centrifuge and aliquots of supernatant were drawn for analysis of [γ-P^{32}] ATP.

The following experimental design was typical: each condition was carried out at least in duplicate. One reaction condition (ΔpH and Ca^{2+}-level) was always done in parallel with at least two control experiments. Most often the first of these was without enzyme but with Ca^{2+} present, and the second was with enzyme and EGTA, but without Ca^{2+}. Occasionally other controls were used: dead enzyme was exchanged for active, and P_i or ADP was omitted from reaction solution.

ATP Detection

ATP synthesis was determined by two different methods. In the first, the incorporation of $^{32}P_i$ into [γ-^{32}P] ATP or glucose-6 [^{32}P]-phosphate was measured by extracting away excess $^{32}P_i$ from the assay

TABLE I

SYNTHESIS OF ATP BY MEANS OF SOLUBILIZED SR PROTEINS, CA^{2+} + MG^{2+} – ATPASE, AND TRYPTIC FRAGMENTS OF SR

Preparation	Set	cpm (test–control)	ATP synthesized	Average of all experiments ±SD	Revised average*	P value
			(nmol/mg min)			
Ca^{2+} + Mg^{2+}-ATPase	1	155	0.285	0.213 ± 0.056	0.230 ± 0.037	P(A,B)
(A)		135	0.248	$N = 7$	$N = 6$	>0.10
		119	0.219			
	2	275	0.244			
		230	0.204			
		126	0.112			
		203	0.180			
R_1w(SR)	1	182	0.335	0.205 ± 0.090	0.174 ± 0.063	P(A,C)
(B)		131	0.241	$N = 5$	$N = 4$	>0.01
		115	0.212			
	2	123	0.109			
		149	0.132			
TD2	1	73	0.134	0.115 ± 0.023	0.115 ± 0.023	P(A,D)
(R_1w 30% fragment A)		72	0.132	$N = 7$	$N = 7$	>0.01
(C)		58	0.107			
	2	154	0.136			
		151	0.134			
		101	0.090			
		84	0.074			
TD2	1	36	0.066	0.062 ± 0.011	0.062 ± 0.011	P(C,D)
(R_1w 10% fragment A)		28	0.052	$N = 5$	$N = 5$	>0.01
(D)		39	0.72			
	2	76	0.067			
		60	0.053			

*Revised average refer to the first two rows (Ca^{2+} + Mg^{2+}-ATPase and R_1w) where the one value far away from the average was excluded.

medium as phosphomolybdate with 2-butanol-benzene (13). After the butanol-benzene extraction a control experiment showed that < 0.01% of the original content of $^{32}P_i$ remained in the aqueous phase.

In the second method 5 μl of the reaction medium was spotted on a plastic-backed TLC sheet coated with polyethylenamine cellulose powder. [γ-P^{32}] ATP was separated from other nucleotides and P_i^{32} by developing the sheet in 0.25 M KH_2PO_4, pH = 3.4. The spots were visualized under short wavelength UV-light and cut out from the sheet and counted. Segments of the TLC sheet and samples of water phase after organic extraction were counted using a 1:1 vol/vol toluene:ethanol scintillation fluid with 2 g/l Omnifluor from J. T. Baker Chemical Co.

Results

The results always showed an internal consistency, e.g., for one condition tested (up to six parallel replication) the control experiments (at least in four parallel replication) were always lower. Different types of control experiments gave the same results. The average counts obtained for P_i^{32} from the experiments were ~ 50% above the average control values. Six different experiments performed under the same condition gave an SD of 31% when the control value was subtracted from the experimental value. The same scattering of results was obtained when six samples were drawn from one test tube for the detection of P^{32} by TLC. The differences of counts per minute between control and test are small, but the differences are statistically significant. That different specific activities of P_i gave proportional counts in independent experiments (Table I) is also taken as an indication of reliability of the results.

It can be noted from Figs. 2 and 3 that the results obtained by organic extraction of excess P_i^{32} from the reaction mixture are consistently lower than the ones obtained by separation on a TLC plate. The difference is expected because of a significant hydrolysis of ATP over time (4). Samples for spotting on the TLC plate were drawn within seconds after the reaction was carried out. The [γ-P^{32}] ATP, which was subject for the other analytical procedure, was exposed to solution for a much longer time (30 min).

The amount of ATP produced via the solubilized enzyme was first investigated with a pH shift of 2.9

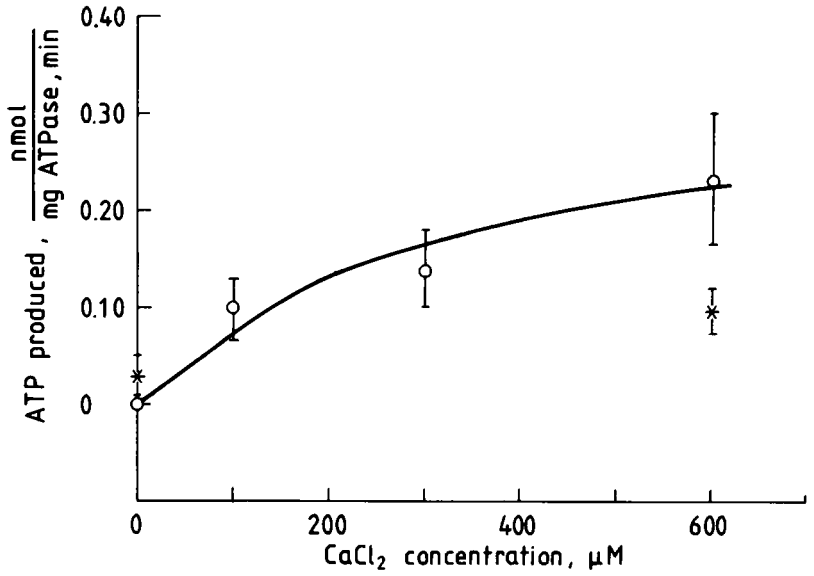

FIGURE 2 ATP synthesis with solubilized Ca^{2+} + Mg^{2+}-ATPase at different levels of $CaCl_2$ by changing pH from 5.1 to 8.0. Reaction conditions are given in Materials and Methods. O, detection of ATP^{32} by TLC; *, detection of ATP^{32} by extraction of excess Pi^{32}.

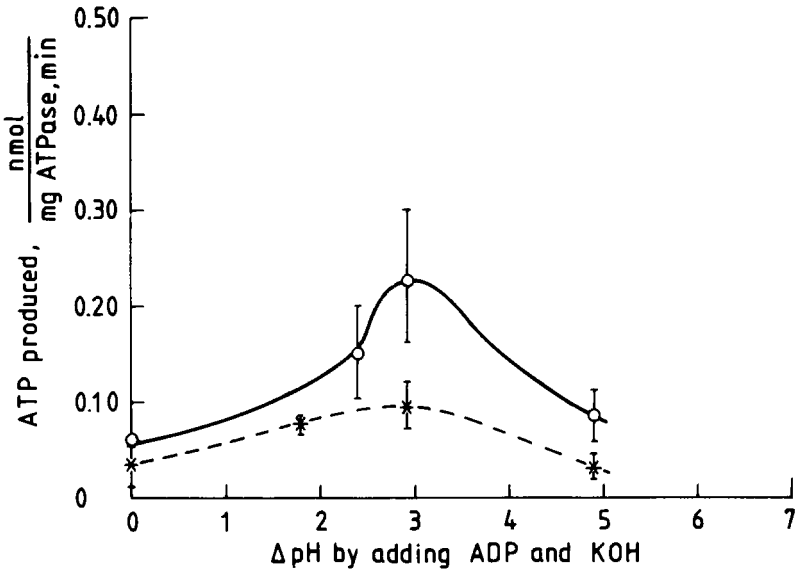

FIGURE 3 ATP synthesis with solubilized Ca^{2+} + Mg^{2+}-ATPase at 0.6 mM $CaCl_2$ for various shifts of pH in reaction medium. Reaction conditions are given in Materials and Methods. O, detection of ATP^{32} by TLC; *, detection of ATP^{32} by extraction of excess Pi^{32}.

units at different constant Ca^{2+}-concentrations from 0 to 0.6 mM. The results are illustrated in Fig. 2 and are given as the difference between experiment and control value. Of the four concentrations of calcium investigated, 0.6 mM gave the highest value. This is the value de Meis and Tume (4) used in their experiment. Higher concentrations will inhibit phosphorylation at pH = 5.1 (4). The value 0.6 mM is still high enough to allow transfer of phosphate from E ~ P to ADP at pH = 8 (4). In subsequent experiments the concentration of 0.6 mM $CaCl_2$ was used in the study of the synthesis as a function of ΔpH. These results are illustrated in Fig. 3. The curve shows an optimum around a ΔpH of ~ 3 units.

The effect of tryptic digestion on the SR was also investigated and the results are given in Table I. The SR used for the tryptic digest was also used in the same experiment as a reference for the synthesis, as was the purified Ca^{2+} + Mg^{2+} ATPase. As expected, the results show that the purified Ca^{2+} + Mg^{2+} ATPase gives a higher value for the ATP synthesized per milligram than the SR vesicles. The results show a correlation between the amount of ATP synthesized and the disappearance of the A_2 fragment which contains the Ca^{2+}-selective transport site in the Ca^{2+} + Mg^{2+}-ATPase (1).

DISCUSSION

The obvious conclusion drawn from our experiment is that the enzyme alone is responsible for the synthesis of ATP. This is because synthesis is measured with the solubilized enzyme and with solubilized tryptically digested enzyme. The observed formation of ATP from E ~ P is probably a one-turnover reaction. The enzyme does not become phosphorylated again after the increased pH.

We observe a maximum synthesis at a pH shift of ~ 3 units. The pH of the solution was changed by injecting ADP, $CaCl_2$, and KOH into the solution from a micropipette. Before proper vortexing is done, there are regions with local pH higher than the final average value for the total solution. Rapid flow mixing equipment may improve this part of the experiment. At higher pH shifts the enzyme is probably denatured.

$\Delta G°$ for the formation of ATP according to the overall reaction

$$ADP + P_i + 2H^+ = ATP + H_2O \quad (1)$$

is 7.3 kcal at pH = 7.0 (14). Eq. 1 alone indicates that the reaction should be favored at low pH's. We find the opposite: a contribution to the synthesis of ATP by a sudden increase of pH of the reaction medium. The reason for this must be that H^+ is acting by modifying the enzyme as a catalyst.

The results from tryptic digestion in Table I show that synthesis of ATP is decreased in parallel with the decrease in Ca^{2+}-uptake as fragment A is cleaved as shown by the gel. Fragment A contains the Ca^{2+} binding site responsible for uptake (10). The data in Table I confirm the reversibility of the uptake reaction. We find values for the synthesis of ATP about one order of magnitude lower than Knowles and Racker (3), who used a Ca^{2+} shift to synthesize ATP. This makes it unlikely that H^+ can substitute for Ca^{2+} in the synthesis mechanism. Instead H^+ must act via another part of the molecule by modifying the Ca^{2+} binding sites. According to de Meis and Tume (4) the affinity of this site for Ca^{2+} increases with increasing pH of the medium. The transfer of phosphate from phosphoenzyme to ADP is dependent upon the saturation of the low affinity Ca^{2+}-binding site, e.g., a fairly high level of Ca^{2+} (40 mM at pH = 6). But this level requirement can be lowered, say, to 0.5 mM by increasing the pH of the medium to 8.0. The conditions for enzyme phosphorylation are not at optimum at $CaCl_2$ 0.6 mM, but a significant part will still be functioning.

Thus there are two enzyme states with a difference in energy that may account for the synthesis of ATP. Each of the states can be obtained by different means, either by changing the Ca^{2+} concentration alone (3), pH alone, as reported here, or by changing both the concentration of Ca^{2+} and H^+ (4). In our in vitro experiment, H^+ plays a regulatory role on the two enzyme-Ca states. The data on tryptic digestion further support that the tryptic digestion results in the disruption of the intimate relationship between the hydrolytic site and the Ca^{2+}-binding sites that is needed for energy transduction.

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