differences in structures, functional roles, or control properties of the microtubules. Resistance to cold-induced depolymerization is one such difference. The significance of the effects of CSF must await the isolation and identification of the CSF molecule and also, possibly, the identification of more subtle behavioral properties of the microtubules which the CSF may modulate.

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Characterization of Medium Inorganic Phosphate–Water Exchange Catalyzed by Sarcoplasmic Reticulum Vesicles†

Masahiro Ariki and Paul D. Boyer*

ABSTRACT: Effects of temperature, Ca^{2+}, and ATP on the extent and characteristics of the medium $P_i \rightleftharpoons HOH$ exchange catalyzed by sarcoplasmic reticulum ATPase are reported. Measurements of the patterns of $[^{18}O]P_i$ species formed from highly labeled $[^{18}O]P_i$ show that a single catalytic pathway is involved in the rapid medium $P_i \rightleftharpoons HOH$ exchange with Mg^{2+} present and the much slower exchange with both Mg^{2+} and Ca^{2+} present. A continued high rate of exchange is observed when ATP concentration is increased up to 5 mM even though the amount of phosphoenzyme formed from P_i is much greater at lower ATP concentrations. This result reveals that binding of ATP in some manner causes a pronounced increase in the rate constant for hydrolysis of the phosphoenzyme. During the rapid oxygen exchange in the absence of Ca^{2+} at 10 and 30 °C, the rate of P_i release from the enzyme·P_i complex is about 5–6 times greater than the rate of phosphoenzyme formation. Both P_i release and phosphoenzyme formation are much slower in the presence of Ca^{2+}, with a greater relative tendency for phosphoenzyme formation, particularly at the lower temperature.

Sarcoplasmic reticulum preparations catalyze a rapid exchange of oxygens between P_i and water (medium $P_i \rightleftharpoons HOH$ exchange)[1] in the presence of Mg^{2+} (Kanazawa & Boyer, 1973). This exchange results from the displacement of an oxygen from phosphate by an aspartyl carboxyl group with resultant formation of a phosphoenzyme, E–P (Boyer et al., 1977). Measurement of the rate of exchange P_i oxygens thus allows evaluation of the rate of the reaction step $E{\cdot}P_i \rightleftharpoons E\text{–}P + HOH$. Additional refinements can give the ratio of this exchange rate to the rate of P_i release (Boyer et al., 1977; Boyer & Ariki, 1980). One purpose of this paper is to report the effects of temperature, ATP, and Ca^{2+} on characteristics of the medium $P_i \rightleftharpoons HOH$ exchange and the relevance of these results to the overall reaction mechanism. Of particular interest is the finding that the presence of 5 mM ATP accelerates hydrolysis of the phosphoryl enzyme intermediate.

A second purpose of this paper makes use of the recently developed recognition that the pattern of $[^{18}O]P_i$ species formed from highly labeled $[^{18}O]P_i$ is indicative of whether single or multiple catalytic pathways are involved in the exchange process. Multiple pathways are revealed by heterogeneous distributions of $[^{18}O]P_i$ species containing 0 to 4 ^{18}O atoms per molecule (Sleep et al., 1978). These studies are of additional interest because of the recent demonstration that in the presence of Ca^{2+} and Mg^{2+} two catalytic pathways of cleavage of ATP by sarcoplasmic reticulum become operative at relatively low ATP concentrations (Boyer & Ariki, 1980). Such modulation by ATP concentration of the rate of reversible E–P formation accompanying net ATP cleavage suggests the possibility of an important role of ATP binding in the overall Ca^{2+} transport process. Results reported herein show that although the medium $P_i \rightleftharpoons HOH$ exchange takes place

† From the Department of Chemistry and the Molecular Biology Institute, University of California at Los Angeles, Los Angeles, California 90024. *Received October 29, 1979.* Supported in part by National Science Foundation Grant PCM 75-18884.

[1] Medium $P_i \rightleftharpoons HOH$ exchange occurs when P_i binds, undergoes exchange, and is released to the medium. Intermediate $P_i \rightleftharpoons HOH$ exchange occurs when P_i formed from ATP incorporates more than one oxygen from water prior to release to the medium.

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in the absence of added ATP, a single catalytic pathway is involved.

Experimental Procedures

Materials. The Ca^{2+} ionophore, A23187, was a gift of Eli Lilly Co. [^{18}O]P_i (99% ^{18}O enrichment) was prepared in this laboratory by Dr. D. D. Hackney (Risley & Van Etten, 1978; Hackney et al., 1979). Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle essentially as described previously (de Meis & Hasselbach, 1971) and stored for several days on ice at 0 °C or lyophilized (Sreter et al., 1970) and stored at −20 °C.

P_i ⇌ HOH, Interpretation and Measurement. The P_i ⇌ HOH exchange was regarded as involving the steps in eq 1,

$$P_i + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}P_i \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{–}P + HOH \qquad (1)$$

with the partitioning between exchange and release steps expressed as the partition coefficient, P_c, where $P_c = k_2/(k_2 + k_{-1})$. The value of P_c may range from 0 to 1 depending on whether few or many exchanges occur each time a P_i binds (Boyer et al., 1977; Hackney & Boyer, 1978).

The extent of total P_i ⇌ HOH exchange was estimated by measuring ^{18}O incorporation from $H^{18}OH$ into P_i initially present or formed during ATP hydrolysis or by ^{18}O loss from [^{18}O]P_i. The P_i was isolated by using an isobutyl alcohol–benzene extraction method (Hackney et al., 1979), and ^{18}O content in P_i was determined by conversion of oxygens to CO_2 and measurement of the mass 46/44 ratio (Boyer & Bryan, 1967) or by conversion of P_i to a volatile phosphate and determination of species with 0 to 4 ^{18}O atoms present per molecule, designated $P^{18}O_0$ to $P^{18}O_4$, as previously described (Hackney et al., 1979; Hackney & Boyer, 1978).

The extent of medium P_i ⇌ HOH exchange was determined by the loss of ^{18}O content in [^{18}O]P_i during the reaction, using the following equation (Boyer & Bryan, 1967): gram-atoms of oxygen exchanged = $4(P_0 + P_t)\ln(1 - F)^{-1}$, where P_0 = mol of P_i initially present, P_t = mol of P_i produced from ATP by sarcoplasmic reticulum ATPase, and F = fraction exchange of isotopic oxygens. This calculation gives a minimal value for the total amount of oxygen exchange occurring, namely, that which would be obtained if P_c values are close to zero. Other approaches are applicable for exchange at higher P_c values (Hackney et al., 1979).

Results

Effect of ATP Concentration on the Medium P_i ⇌ HOH Exchange in the Presence of Ca^{2+}. Although ATP has been shown to induce an exchange of oxygens of P_i with water by sarcoplasmic reticulum vesicles in the presence of Ca^{2+} (de Meis & Boyer, 1978), data on ATP concentration effects and on the extent of the stimulation have not been reported. Results of experiments for such a purpose are given in Table I.[2] For these experiments, measurements were made of the loss of ^{18}O from medium [^{18}O]P_i, a convenient way to measure medium P_i ⇌ HOH exchange without interference of intermediate P_i ⇌ HOH exchange.

The data in Table I confirm that a pronounced increase in medium P_i ⇌ HOH exchange is induced by ATP, and, further, they show that 50 μM ATP is as effective as 5 mM ATP. The acceleration of the medium P_i ⇌ HOH exchange by cleavage of ATP in the presence of Ca^{2+} is over 20-fold.

[2] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

Table I: Effect of ATP Concentration on the Medium P_i ⇌ HOH Exchange in Presence or Absence of Ca^{2+} [a]

Ca^{2+} (μM)	EGTA (mM)	ATP (mM)	incubation time (min)	P_i formed (mM)	medium P_i ⇌ HOH exchange [b] (μg-atoms min^{-1} mg^{-1})	approximate E–P from P_i [c] (μmol/g)
125	0	5	1	1.14	1.88	0.03
		0.05	3	0.83	1.91	1.00
		0	20	0	0.08	0.21
0	1.25	5	1	<0.1	1.97	
		0.05	3	<0.1	3.83	
		0	5	0	6.32	1.21

[a] The reaction mixture in a final volume of 2 mL at pH 6.5 and 30 °C contained 20 mM Tris-maleate buffer, 20 mM $MgCl_2$, 5 mM phosphoenolypyruvate, 100 μg of pyruvate kinase, 10 mM KCl, 0.94 mg of intact vesicle protein, 10 μg of A23187, 125 μM $CaCl_2$ or 1.25 mM EGTA,[2] 5 mM [^{18}O]P_i containing 1% ^{18}O, and 5 mM, 0.05 mM, or no ATP. The reaction was initiated by the addition of [^{18}O]P_i and ATP. After incubation for the times indicated, the reaction was quenched with 0.5 mL of 2 N perchloric acid and the ^{18}O retained in P_i determined by conversion of phosphate oxygens to CO_2. [b] Minimum amount of total exchange of oxygen atoms between HOH and P_i; see Experimental Procedures. [c] Values taken from experiments run under similar conditions but with 150 μM Ca^{2+} (when Ca^{2+} present) and with 10 mM $^{32}P_i$ (de Meis & Boyer, 1978).

Also shown in Table I is the effect of ATP on medium P_i ⇌ HOH exchange in the absence of Ca^{2+}. The rapid exchange is inhibited about 3-fold by 5 mM ATP. Both ATP and ADP inhibition have been reported earlier (Kanazawa & Boyer, 1973).

Characterization of the Medium P_i ⇌ HOH Exchange by [^{18}O]P_i Species Measurements. For these experiments the Mg^{2+}-activated exchange was carried out with P_i highly labeled with ^{18}O, with and without Ca^{2+} addition. Results of an experimental series at 30 °C are given in Table II. With Ca^{2+} present, the exchange was much slower; the vesicle concentration was 50 times greater than that used when only Mg^{2+} was present. Semilog plots of the decreases in average total ^{18}O content and of the $P^{18}O_4$ species against time were linear; such linearity shows a first-order loss, as expected if the oxygen-exchange rates remained constant with time.

Also given in Table II are the theoretical values for the expected [^{18}O]P_i species if only one catalytic route for exchange was operative, with the observed and theoretical values for the $P^{18}O_4$ species set equal. The close agreement between observed and theoretical values shows that only one exchange process is occurring, characterized by a P_c close to 0.22 in the presence of and 0.13 in the absence of Ca^{2+}.

Effect of Temperature on the Medium P_i ⇌ HOH Exchange. To assess the effect of temperature on the exchange rates and P_c values in the presence and absence of Ca^{2+}, experiments as reported in Table II were repeated at 10 instead of 30 °C. Results are given in Table III. Again, losses of total ^{18}O and $P^{18}O_4$ species were first order and the exchanges are consistent with a single catalytic pathway with one characteristic P_c.

Table IV summarizes the rate data and P_c values for this series of experiments. Decrease in temperature has a greater effect on the exchange rate in the absence of Ca^{2+}. Also, the Ca^{2+}-activated exchange shows a more marked shift in P_c with lowering of temperature than the much faster exchange in the absence of Ca^{2+}.

Discussion

The results reported in this paper allow further characterization of the most rapid reaction catalyzed by sarcoplasmic

Table II: Distribution of [^{18}O]P_i Species during Medium $P_i \rightleftharpoons$ HOH Exchange at 30 °C[a]

Ca^{2+} (μM)	time (min)	total ^{18}O content (% ^{18}O)	% of total $P^{18}O_0$	$P^{18}O_1$	$P^{18}O_2$	$P^{18}O_3$	$P^{18}O_4$
104	0	98.2	0.9	0.0	0.1	3.0	96.0
			(0.9)	(0.0)	(0.1)	(3.0)	(96.0)
	30	89.8	1.1	1.4	5.6	21.2	70.7
			(1.0)	(1.3)	(6.1)	(20.7)	(70.9)
	50	83.8	1.0	2.6	10.7	31.7	54.0
			(1.3)	(3.0)	(11.2)	(28.3)	(56.3)
	120	68.3	3.1	10.1	24.5	35.2	27.1
			(3.3)	(10.7)	(24.0)	(33.7)	(28.4)
	200	54.5	7.8	20.7	31.3	26.1	14.1
			(7.8)	(20.7)	(30.7)	(27.5)	(13.3)
0	0	98.5	0.9	0.0	0.1	2.6	96.4
			(0.9)	(0.0)	(0.1)	(2.6)	(96.4)
	30	87.5	1.1	1.5	6.8	27.2	63.4
			(1.0)	(1.3)	(7.5)	(27.1)	(63.1)
	80	73.7	2.6	6.7	19.6	37.0	34.1
			(1.9)	(6.5)	(20.6)	(37.0)	(34.0)
	120	63.6	3.9	12.5	28.8	34.9	19.9
			(3.6)	(13.1)	(28.8)	(34.7)	(20.0)
	200	52.0	9.6	22.4	31.5	23.3	13.2
			(7.7)	(22.6)	(33.5)	(26.6)	(9.7)

[a] The reaction solution contained in a final volume of 1 mL at pH 6.5 and 30 °C 5 mM Mes buffer, 20 mM $MgCl_2$, 50 mM KCl, 0.5 mM EGTA, 0.6 mM $CaCl_2$ (with Ca^{2+}) or no $CaCl_2$ (no Ca^{2+}), 5 μg of A23187/mL, 0.4 mg of intact vesicles protein/mL (with Ca^{2+}), 20 μg of intact vesicles protein/mL (no Ca^{2+}), and 5 mM [^{18}O]P_i (99%). Reaction was initiated with 0.1 mL of 50 mM [^{18}O]P_i. At indicated time intervals 0.1-mL aliquots were quenched by vortexing with 2 mL of 20 mM Tris containing 50 μL of $CHCl_3$ for 1 min and assayed as indicated under Experimental Procedures. Numbers in parentheses are theoretical values expected for one catalytic pathway for exchange (Sleep et al., 1978).

Table III: Distribution of [^{18}O]P_i Species during Medium $P_i \rightleftharpoons$ HOH Exchange at 10 °C[a]

Ca^{2+} (μM)	time (h)	total ^{18}O content (%)	% of total $P^{18}O_0$	$P^{18}O_1$	$P^{18}O_2$	$P^{18}O_3$	$P^{18}O_4$
104	0	98.5	0.4	0.2	0.2	3.3	95.9
			(0.4)	(0.2)	(0.2)	(3.3)	(95.9)
	4	93.9	0.9	1.3	2.5	11.9	83.4
			(0.9)	(1.7)	(3.6)	(8.8)	(85.1)
	22	76.5	5.4	8.2	12.7	22.6	51.1
			(4.1)	(9.1)	(14.7)	(20.9)	(51.2)
	30	69.7	7.7	11.3	16.1	24.4	40.5
			(6.3)	(12.5)	(18.0)	(22.5)	(40.7)
0	0	97.6	0.8	0.6	0.6	2.5	95.5
			(0.8)	(0.6)	(0.6)	(2.5)	(95.5)
	4	96.4	0.4	0.3	1.2	9.6	88.5
			(0.8)	(0.7)	(1.3)	(6.5)	(90.7)
	22	86.7	0.9	1.9	7.8	28.4	61.0
			(1.0)	(2.2)	(8.3)	(25.8)	(62.6)
	30	81.8	1.8	3.6	11.7	31.6	51.3
			(1.3)	(3.7)	(12.7)	(31.3)	(51.1)

[a] Reaction conditions are as given in Table II, except the temperature was 10 °C. Numbers in parentheses are theoretical values expected for one catalytic pathway for exchange (Sleep et al., 1978).

reticulum ATPase, namely, the exchange of phosphate oxygens with water in the presence of Mg^{2+} and absence of Ca^{2+} (Kanazawa & Boyer, 1973). The analyses of [^{18}O]P_i species formed from highly labeled P_i (Table IV) show that of the two steps involved in this exchange (eq 1), the binding and release of P_i at 30 °C is some 7 to 8 times more rapid than the

Table IV: Kinetic Parameters Obtained from Medium $P_i \rightleftharpoons$ HOH Exchange Catalyzed by Sarcoplasmic Reticulum Vesicles at 10 and 30 °C

temp (°C)	$CaCl_2$ (mM)	EGTA (mM)	rate: total ^{18}O loss (ng-atom of oxygen min^{-1} mg^{-1})	rate: loss of $P^{18}O_4$ (nmol min^{-1} mg^{-1})	P_c[a]	no. of reversals[b]
30	0.6	0.5	160	134	0.22	0.28
	0	0.5	3660	3300	0.13	0.15
10	0.6	0.5	9.60	5.95	0.50	1.01
	0	0.5	98.4	86.0	0.16	0.19

[a] The partition coefficient, P_c, was calculated from the equation $3P_c = 4(1 - k_4/k_t)$ where k_4 = the rate of loss of $P^{18}O_4$ species and k_t = the rate of total ^{18}O loss (Hackney & Boyer, 1978). [b] The average number of reversals, R, of $E{\cdot}P_i$ formation from E–P prior to P_i release from $E{\cdot}P_i$ was calculated from the relation $R = P_c/1 - P_c$ (Boyer et al., 1977).

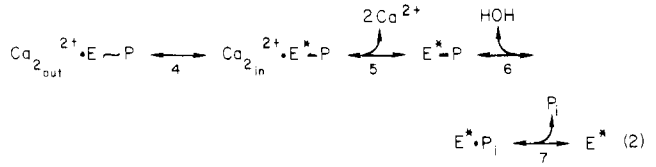
formation of E–P from $E{\cdot}P_i$. Further, the pattern of [^{18}O]P_i species formed as exchange proceeds (Table II) shows that, within experimental error, all the exchange proceeds as if a single catalytic pathway is involved. In addition, the much slower $P_i \rightleftharpoons$ HOH exchange demonstrable in the presence of 125 μM Ca^{2+} (<5% of the rate without Ca^{2+}) occurs with a P_c characteristic of a single catalytic pathway.

The finding that the medium $P_i \rightleftharpoons$ HOH exchange in the presence or the absence of Ca^{2+} occurs by a single catalytic pathway is of additional interest because of the recent demonstrations of modulation by ATP concentration of the intermediate $P_i \rightleftharpoons$ HOH exchange accompanying ATP cleavage. A single pathway appears to operate at higher ATP concentrations, but as the ATP concentration is lowered there is involvement of two pathways, one with a relatively high P_c (Boyer & Ariki, 1980). Of these two pathways, only the one with the lower P_c value appears to be accessible to P_i in the absence of ATP cleavage. Such results suggest that at low ATP the E–P that is formed from ATP more readily undergoes reversible hydrolytic cleavage than the E–P arising from P_i. The possible nature of these forms of E–P and the modulation of the catalytic routes by ATP concentration are under current investigation in our laboratory.

Perhaps the most interesting feature of our results is the demonstration of prominent modulation by higher levels of ATP of the reaction steps of E–P cleavage and P_i release. As summarized in Table I, an increase in ATP concentration from 0 to 50 μM in the presence of 125–150 μM Ca^{2+} and 5–10 mM P_i results in the presence of more E–P formed from P_i; with further increase of ATP to 5 mM a marked drop in the level of E–P formed from P_i results. It has been shown that under these conditions (5 mM ATP) considerable E–P (about 2.6 μmol/g of protein) formed from ATP is present (de Meis & Boyer, 1978). However, only the E–P formed from P_i can participate in the medium $P_i \rightleftharpoons$ HOH exchange. Thus, with 5 mM ATP present, the low level of E–P must be hydrolyzing and yielding P_i some 20 to 30 times more rapidly than with 50 μM ATP present.

These observations need evaluation in terms of a reaction sequence as developed from previous data in the field. In such a sequence (de Meis & Boyer, 1978), the release steps from E–P may be as depicted in eq 2.

With 5 mM ATP, 125 μM Ca^{2+}, and 10 mM P_i, most of the E–P is likely present as the $Ca_2^{2+}{\cdot}E^*$–P form (Verjovski-Almeida & Inesi, 1979) with the phosphoryl group arising from ATP (de Meis & Boyer, 1978). Conversion to



the E*–P form must contribute considerably to rate limitation of overall ATP cleavage. This could involve steps besides just the Ca^{2+} release depicted by step 5 of eq 2. The hydrolysis of E–P in step 6 is markedly accelerated by the presence of 5 mM ATP in the medium. The ATP binding that drives this step could be that responsible for the well-documented secondary increase in the rate of ATP cleavage produced by millimolar ATP concentrations (Yamamoto & Tonomura, 1967; Inesi et al., 1967) and attributed in recent reports to a control site on the enzyme (Dupont, 1977; Taylor & Hattam, 1979).

The marked decrease in the rate of the medium $P_i \rightleftharpoons HOH$ exchange with a decrease in temperature from 30 to 10 °C (Table I) is in harmony with results of others showing that the overall process of ATP cleavage and of Ca^{2+} transport is sharply inhibited by temperature decrease (Masuda & de Meis, 1977). A variety of observations suggest that on lowering the temperature a transition occurs near 18 °C, resulting in a decrease in mobility and changes in kinetic parameters (Madeira et al., 1974; Lee et al., 1974; Madeira & Antunes-Maderia, 1975; Davis et al., 1976; Hidalgo et al., 1976; Anzai et al., 1978). These likely result from additional restraints being imposed on protein conformational changes accompanying the process. Our data allow some conclusions about differential effects on specific rate constants. Temperature decrease does not significantly change the value of P_c in the absence of Ca^{2+}, while the value is increased more than twice by a temperature drop from 30 to 10 °C in the presence of Ca^{2+}. Kanazawa reported that k_2 in eq 1 decreases markedly with the lowering of temperature in the absence of Ca^{2+}, meaning that k_2 is affected more than k_{-2} (Kanazawa, 1975).

At 10 °C as at 30 °C the distribution of $[^{18}O]P_i$ species is characteristic of that expected if a single catalytic pathway were operative. All enzyme molecules appear to be similarly constrained at the lower temperature.

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