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Vacuolar ATPases, like F_1,F_0-ATPases, show a strong dependence of the reaction velocity on the binding of more than one ATP per enzyme

(**^{18}O exchange/cooperativity/ATP synthase/binding-change mechanism**)

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ABSTRACT **Recent studies with vacuolar ATPases have shown that multiple copies catalytic subunits are present and that these have definite sequence homology with catalytic subunits of the F_1,F_0-ATPases. Experiments are reported that assess whether the vacuolar ATPases may have the unusual catalytic cooperativity with sequential catalytic site participation as in the binding change mechanism for the F_1,F_0-ATPases. The extent of reversal of bound ATP hydrolysis to bound ADP and P_i as medium ATP concentration was lowered was determined by ^{18}O-exchange measurements for yeast and neurospora vacuolar ATPases. The results show a pronounced increase in the extent of water oxygen incorporation into the P_i formed as ATP concentration is decreased to the micromolar range. The F_1,F_0-ATPase from neurospora mitochondria showed an even more pronounced modulation, similar to that of other F_1-type ATPases. The vacuolar ATPases thus appear to have a catalytic mechanism quite analogous to that of the F_1,F_0-ATPases.**

Recently, several different types of experimental observations have suggested that vacuolar ATPases may be mechanistically similar to the F_1,F_0-ATPases.‡ These are (*i*) demonstration that binding of *N,N'*-dicyclohexylcarbodiimide (DCCD) to a small proteolipid subunit blocks catalytic activity (1–3) and that reaction of DCCD with only one of the multiple copies of the subunit suffices for inhibition (3); (*ii*) demonstration of a high-affinity catalytic site during single-site catalysis (4); (*iii*) demonstration that an ≈70-kDa subunit (5, 6), which probably carries a catalytic site, and an ≈60-kDa subunit (7) have definitive sequence homology with the α and β subunits of the F_1,F_0-ATPases; and (*iv*) demonstration that the ≈60- to ≈70-kDa subunits are present in multiple copies (4, 8, 9), probably three per enzyme (10), and that about six copies of the DCCD-reactive subunit and single copies of other subunits are probably present (10). These similarities of vacuolar and F_1,F_0-ATPases in structure and single-site properties made it important to determine whether the vacuolar F_1-F_0 and the ATPases show similar catalytic behavior during net ATP hydrolysis.

A striking catalytic property of F_1,F_0-ATPases is the pronounced catalytic cooperativity between nucleotide binding sites. One important experimental result documenting this cooperativity is the demonstration that as the ATP concentration is lowered, the extent of incorporation of water oxygens into each P_i released increases markedly. Furthermore, when [γ-^{18}O]ATP is cleaved, the distribution of ^{18}O in the P_i formed shows that only one catalytic pathway operates at different ATP concentrations. As the medium ATP concentration is lowered, ATP bound at a catalytic site continues to undergo reversible cleavage to bound ADP and P_i until ATP binds at another site (11, 12). This ^{18}O-exchange approach has now been applied to the ATPases from vacuolar membranes of neurospora and yeast. The results demonstrate that the vacuolar ATPases show cooperative interactions analogous to the F_1,F_0-ATPases.

MATERIALS AND METHODS

Chemicals. Phenylmethylsulfonyl chloride was obtained from Fluka; sorbitol, Ficoll 400, dithiothreitol, and ADP were purchased from Sigma. ^{18}O-enriched P_i and phospho*enol*-pyruvate were prepared from highly enriched water as described (13).

ATPase Preparations. Yeast vacuoles *Saccharomyces cerevisiae* were kindly provided by T. Melese (Columbia University) and were obtained as a by-product when nuclear membranes were isolated essentially as described by Aris and Blobel (14). Vacuoles floating in a Ficoll layer (10 ml) were suspended in 1.0 M sorbitol (100 ml) containing 20 mM Hepes-NaOH buffer (pH 7.4) and 0.5 mM phenylmethylsulfonyl chloride. After centrifugation at 90,000 × *g* for 30 min, the vacuolar pellet was resuspended in the same buffer and used for further analysis. This preparation contained traces of the plasma membrane ATPase, F_1-ATPase, and pyrophosphatase.

Vesicles containing vacuolar ATPase from *Neurospora crassa* were purified according to the last version of the method described by Bowman and Bowman (15). The preparation of vacuoles was not reproducible in different experiments and considerable F_1-ATPase was present (up to 70% of the total ATPase activity). Vesicles obtained from vacuoles contained much less F_1-ATPase activity and were suitable for the ^{18}O experiments. The total ATPase activity was inhibited up to 45% by 0.1 M KNO_3 and 1 mM NaN_3 caused 20% inhibition. The sample contained traces of the plasma membrane ATPase and pyrophosphatase. Specific activity of the vacuolar ATPase was 2.7 μmol/ (min × mg) at 37°C.

For tests with F_1,F_0-ATPase, mitochondria were isolated from *N. crassa* cells (15) and submitochondrial particles were made by sonication. The submitochondrial particles at pH 7.5 in 10 mM Tris·HCl buffer, containing 0.25 M sucrose, 0.5 mM EDTA, and 0.5 mM dithiothreitol, were centrifuged at 90,000 × *g* for 45 min, resuspended in the same volume of buffer and centrifuged again. The final pellet was resuspended in the same buffer and stored at 4°C for no more than a day before use. The specific activity at a protein concentration of 5 mg/ml, assayed as described, was 6.6 μmol/(min × mg) at

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‡The designations F_1,F_0-ATPase and ATP synthase refer to the same enzyme. The first designation is used for this paper, in which only the ATPase function is being studied.

37°C. The activity was inhibited 93% by 1 mM NaN_3, as expected for catalytic activity of an ATP synthase.

ATP Hydrolysis and ^{18}O-Exchange Measurements. All ATP hydrolysis measurements were made with 10 μM carbonylcyanide *m*-chlorophenylhydrazone present to dissipate any membrane potential. All oxygen-exchange experiments with vacuolar ATPase were done in the presence of 1 mM NaN_3 and 20 μM vanadate to inhibit any mitochondrial or plasma membrane ATPase. Duplicate incubations with yeast vacuoles were made at pH 7.2 in a 2-ml vol containing 50 mM Hepes-KOH, 3 mM $MgCl_2$, 0.5 mM dithiothreitol, pyruvate kinase (100 μg/ml), 1 mM [^{18}O]phospho*enol*pyruvate, and 10 or 100 μM ATP. Sufficient vacuoles were added to hydrolyze at least 40 nmol of ATP when ATP was 10 μM and >100 nmol at higher ATP concentrations in 30 min at 30°C. Highly enriched [^{18}O]P_i (100 nmol) was added to all samples just before quenching the reaction with chloroform. In the control samples, the same amount of vacuoles was added after the chloroform quench. Dilution of the $P^{18}O_4$ isotopomer gave a measure of P_i present in control samples and P_i generated by enzymic hydrolysis. In separate experiments, baker's yeast pyrophosphatase was added to 100 nmol of highly enriched [^{18}O]P_i in the same reaction medium to check whether the rapid oxygen exchange catalyzed by this enzyme, if present, might influence the results. At least 10 times more pyrophosphatase activity was added than was present in the vacuoles used for ATP hydrolysis. No significant P_i oxygen exchange was found under these conditions, giving assurance that any pyrophosphatase in the vacuolar ATPase preparations was not influencing our results.

Oxygen-exchange experiments with vacuolar ATPase and with the F_1,F_0-ATPase in neurospora submitochondrial particles were done as with baker's yeast enzyme but with some modifications. Tris·HCl buffer (0.05 M; pH 7.5) was used instead of Hepes and 3 mM [^{18}O]phospho*enol*pyruvate was used when the ATP concentration was >0.5 mM. Purified pyrophosphatase from *N. crassa* (specific activity, 400 units/mg) was used to check whether medium P_i exchange might have influenced the final distribution of the P_i isotopomers. No evidence for interference was found.

Analysis of ^{18}O-Exchange Data. Purification of the P_i, derivatization, and analysis were performed as described (13). Distributions of [^{18}O]P_i in the control samples were subtracted from the distributions in the samples where enzymic hydrolysis took place. The final observed distributions of [^{18}O]P_i species were analyzed for how well they fit the predicted distributions for one or two catalytic pathways (16). If only one pathway prevails, the results will agree with distributions predicted for a single P_c value, where P_c is the probability that bound P_i will re-form bound ATP before it is released. The sum of the absolute differences between observed and predicted distributions for all four P_i isotopomers was calculated for each ATP concentration. The data were statistically processed by the least-squares method for one or two pathways to give an observed Δ%, which provides a measure of how well the predicted and observed distributions agree (17). As a means of evaluating the significance of differences, the Δ% values were calculated for the variations of the duplicates against the average distribution for each ATP concentration. The variations of duplicates gave a mean value of 0.97% with a variation coefficient of ±0.43 for the experiments with F_1,F_0-ATPase and 1.28% ± 0.86% with vacuolar ATPase from *N. crassa*.

RESULTS

ATP modulation of the extent of reaction reversals during hydrolysis of ATP by the vacuolar ATPase from *N. crassa* was tested over a range from 1 to 500 μM ATP. A constant level of [γ-^{18}O]ATP was maintained by using [^{18}O]phospho*enol*pyruvate and pyruvate kinase. The original distribution of [^{18}O]P_i isotopomers formed from the [^{18}O]P_i in phospho*enol*pyruvate was measured after acid hydrolysis in the presence of $HgCl_2$ (13). The distribution of [^{18}O]P_i isotopomers formed by the ATPase at different ATP concentrations is presented in Table 1. The table also gives the best matching distribution of [^{18}O]P_i isotopomers calculated for a homogeneous reaction pathway proceeding with one P_c value and the Δ% values for each ATP concentration. Comparison of the observed distribution with the best fit for a distribution with one P_c shows that the matching is quite satisfactory (Δ% average = 1.69 ± 0.82 compared to ±0.97 for precision of duplicates). Predicted distributions are thus within 1% of the observed distributions.

Table 1. ^{18}O-exchange parameters for neurospora vacuolar ATPase

ATP, μM	Source of data	[^{18}O]P_i isotopomers, %				P_c for calc. values	Δ%
		$P^{18}O_0$	$P^{18}O_1$	$P^{18}O_2$	$P^{18}O_3$		
500	Obs.	1.91	9.15	39.81	49.13		
	Calc.	1.22	10.41	38.95	49.42	0.17	3.1
100	Obs.	2.25	11.20	39.69	46.97		
	Calc.	1.50	11.68	39.67	47.16	0.22	1.5
50	Obs.	2.54	13.95	41.36	42.16		
	Calc.	2.36	14.59	40.60	42.45	0.32	1.9
10	Obs.	9.74	24.38	37.57	28.31		
	Calc.	9.29	24.74	38.00	27.96	0.58	1.6
5	Obs.	22.96	30.64	29.70	16.70		
	Calc.	23.34	29.63	29.68	17.35	0.75	2.1
1	Obs.	67.77	17.10	10.58	4.55		
	Calc.	67.80	17.50	10.13	4.57	0.94	1.0

The isotopomers with 0–3 ^{18}O present are designated $P^{18}O_0$–$P^{18}O_3$. Δ% gives statistical measure of how well the calculated and observed values agree.

Similar results were obtained for the vacuolar ATPase from baker's yeast, where only two ATP concentrations were checked. The data are presented in Table 2. To test whether the vacuolar ATPase was responsible for P_i production at low ATP concentrations, the sensitivity of the enzymic hydrolysis at 10 μM ATP to KNO_3 was assessed. With 100 mM nitrate present activity was inhibited by 31%. This is less than the ≈60% inhibition produced by 100 mM nitrate at higher ATP concentration with the purified vacuolar ATPase from neurospora; however, 100 mM nitrate did not inhibit the plasma membrane or the mitochondrial ATPase of neurospora (18). Furthermore, the data of Table 2 show that all the P_i was produced by one catalytic pathway, and thus it is highly unlikely that more than one ATPase was operative. This result, the nitrate sensitivity, and the mode of preparation give reasonable assurance that the properties of the vacuolar ATPase are being observed.

For comparative purposes, the ATP modulation of ATP hydrolysis by the F_1,F_0-ATPase present in submitochondrial particles of *N. crassa* was assessed. The parameters from the ^{18}O-exchange measurements are presented in Table 3.

The values for the number of water oxygens appearing in each P_i formed by the F_1,F_0-ATPase and the vacuolar ATPase from neurospora are shown in Fig. 1. The depen-

Table 2. ^{18}O-exchange parameters for yeast vacuolar ATPase

ATP, μM	Source of data	[^{18}O]P_i isotopomers, %				P_c for calc. values	Δ%
		$P^{18}O_0$	$P^{18}O_1$	$P^{18}O_2$	$P^{18}O_3$		
100	Obs.	1.11	7.79	38.03	53.07		
	Calc.	0.89	8.57	37.42	53.12	0.09	1.7
10	Obs.	5.30	19.62	41.81	33.27		
	Calc.	5.26	20.38	40.21	34.15	0.47	3.3

See Table 1 legend for details.

Table 3. ATP modulation of ATP hydrolysis by F_1,F_0-ATPase from neurospora mitochondria

ATP, μM	Source of data	[^{18}O]P_i isotopomers, %				P_c for calc. values	Δ%
		$P^{18}O_0$	$P^{18}O_1$	$P^{18}O_2$	$P^{18}O_3$		
2000	Obs.	0.66	7.11	35.41	56.82		
	Calc.	0.69	7.02	35.47	56.82	0.01	0.2
500	Obs.	1.08	9.44	38.22	51.26		
	Calc.	1.03	9.46	38.24	51.27	0.13	0.1
100	Obs.	2.72	14.56	40.00	42.73		
	Calc.	2.31	14.46	40.58	42.64	0.31	1.2
50	Obs.	4.35	19.05	40.42	36.18		
	Calc.	4.36	18.97	40.56	36.11	0.44	0.3
20	Obs.	12.07	26.38	36.97	24.59		
	Calc.	12.02	26.57	36.34	25.06	0.63	1.4
10	Obs.	20.12	28.86	32.48	18.54		
	Calc.	19.94	29.25	31.60	19.21	0.73	2.1
5	Obs.	40.58	27.43	21.13	10.87		
	Calc.	40.56	27.67	21.03	10.74	0.85	0.5

See Table 1 legend for details.

dencies on the ATP concentration are somewhat different for the two ATPases, but both show a sharp upswing in the extent of water oxygen incorporation into P_i as the ATP concentration is lowered. The water oxygen incorporation into the P_i formed at the lowest ATP concentration for the vacuolar ATPase corresponds to an average of 17 reversals of bound ATP hydrolysis before P_i release. More reversals would be expected at lower ATP concentrations. The mitochondrial F_1-ATPase from beef heart, when tested at ATP concentrations lower than those for the experiments of Fig. 1, showed >300 reversals before P_i release (12).

DISCUSSION

The F_1,F_0-ATPases are unusual among enzymes because of the presence of three copies of catalytic subunits and single copies of some other subunits and their striking degree of catalytic cooperativity, such that product release by one catalytic subunit depends on substrate binding to a second catalytic subunit (19). A prominent expression of this cooperativity is shown by the ATP modulation of the exchange of phosphate oxygens with water oxygens during net ATP hydrolysis. As the ATP concentration is lowered, the number of water oxygens incorporated into each P_i formed by the mitochondrial F_1-ATPase from beef heart increases from slightly above the 1.0 required by the reaction stoichiometry to nearly 4 (12). This marked increase in oxygen exchange is as expected if product release cannot occur until substrate binds at another catalytic site and represents >300 reversals of hydrolysis of bound ATP to bound ADP and P_i before the P_i is released. The results in this paper show that a similar increase in oxygen exchange as the ATP concentration is lowered occurs with the vacuolar ATPases.

Other important deductions come from measurements of the distributions of [^{18}O]P_i isotopomers formed from [γ-^{18}O]ATP hydrolysis by vacuolar ATPases. At ATP concentrations above and below those necessary for half-maximum velocity, the distributions concur with those expected if only one catalytic pathway is involved at a given ATP concentration, again like the F_1,F_0-ATPases. This shows that the modulation by ATP is not due to changes in the proportions of enzyme forms, one giving little and one giving much oxygen exchange.

These findings demonstrate that with the vacuolar ATPases, the binding and/or reaction of more than one ATP per enzyme is necessary to obtain any appreciable rate of net catalysis and that all participating catalytic sites have similar properties. This finding is in harmony with the report that the vacuolar ATPase from yeast has a catalytic site occupied at

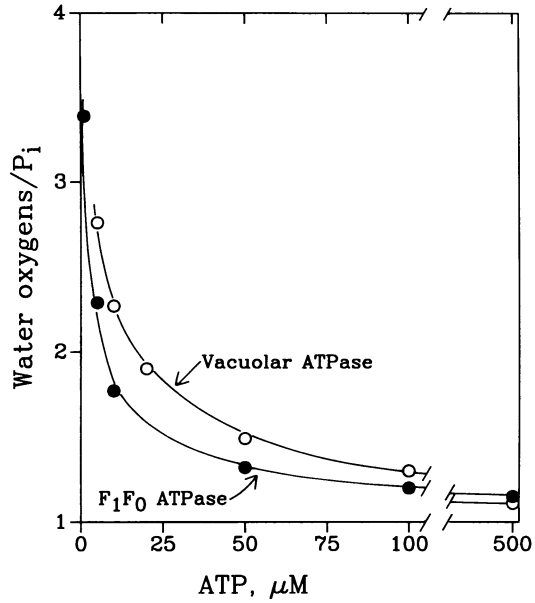

FIG. 1. Comparison of the ATP modulation of oxygen exchange by vacuolar ATPase and F_1,F_0-ATPase from neurospora.

ATP concentrations far below that necessary for half-maximum velocity (4), a prominent characteristic of the F_1,F_0-ATPases (20). Such results, together with the structural analogies mentioned in the Introduction, make it highly likely that the two types of ATPases share common mechanistic features. This would mean that the F_1,F_0-ATPases are no longer alone among enzymes in showing catalytic cooperativity with alternating participation of catalytic sites.

It does need to be recognized, however, that the oxygen-exchange data and the slow single-site catalysis data by themselves do not establish a pattern of catalytic cooperativity for vacuolar ATPases. For example, these observations could be explained by a direct replacement action, in which the dissociation of ADP and P_i occurs in more than one step. When partial dissociation occurs, partial insertion of medium ATP into the binding site could favor further dissociation of the bound products. With the F_1,F_0-ATPases, the additional evidence favoring sequential catalytic site participation, as in the binding mechanism, is substantial. This includes the patterns of exchange of ^{32}P- and ^{18}O-labeled substrates (21–23), the demonstrations that three catalytic binding sites are present with strong negative cooperativity of binding (24–26), the participation of more than one site in steady-state catalysis (27, 28), initial velocity and ^{18}O-exchange data giving evidence for participation of three nucleotide binding sites as substrate concentration is increased with increasing dissociation constants (20, 29, 30), demonstration that three sites have the potential for catalytic activity (16), the strong inhibition of catalytic activity by derivatization of only one of the three catalytic subunits (see ref. 31 and references therein), and properties of mutants with normal and defective subunits that point to the need for three normal subunits for activity (32, 33). Additional evaluations with the vacuolar ATPases along the lines of the above investigations will be essential to find how closely they resemble the F_1,F_0-ATPases in reaction mechanism.

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