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F_1 ATPase from the Thermophilic Bacterium PS3 (TF_1) Shows ATP Modulation of Oxygen Exchange†

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Received March 10, 1989; Revised Manuscript Received May 10, 1989

ABSTRACT: The ATPase from the ATP synthase of the thermophilic bacterium PS3 (TF_1), unlike F_1 ATPase from other sources, does not retain bound ATP, ADP, and P_i at a catalytic site under conditions for single-site catalysis [Yohda, M., & Yoshida, M. (1987) *J. Biochem. 102*, 875–883]. This raised a question as to whether catalysis by TF_1 involved alternating participation of catalytic sites. The possibility remained, however, that there might be transient but catalytically significant retention of bound reactants at catalytic sites when the medium ATP concentration was relatively low. To test for this, the extent of water oxygen incorporation into P_i formed by ATP hydrolysis was measured at various ATP concentrations. During ATP hydrolysis at both 45 and 60 °C, the extent of water oxygen incorporation into the P_i formed increased markedly as the ATP concentration was lowered to the micromolar range, with greater modulation observed at 60 °C. Most of the product P_i formed arose by a single catalytic pathway, but measurable amounts of P_i were formed by a pathway with high oxygen exchange. This may result from the presence of some poorly active enzyme. The results are consistent with sequential participation of three catalytic sites on the TF_1 as predicted by the binding change mechanism.

The sequential participation of two or three catalytic sites during ATP hydrolysis by the F_1 ATPase leads to the prediction that when the medium ATP concentration is lowered a catalytic site should release bound ADP and inorganic phosphate (P_i)[1] slowly until another ATP binds at another catalytic site. Thus, if medium ATP is removed, or if the enzyme is exposed to substoichiometric amounts of ATP, tightly bound ATP, ADP, and P_i should be present at a catalytic site. Experimental evidence for this important characteristic was first reported by Moudrianakis (Adolfsen & Moudrianakis, 1976) for the enzyme from the bacterium *Alcaligenes faecalis* and has since been demonstrated for the mitochondrial (Gresser et al., 1982; Cross et al., 1982; Penefsky, 1985; Cunningham & Cross, 1988), chloroplast (Wu & Boyer, 1986), yeast (Konishi et al., 1987), and *Escherichia coli* (Wise et al., 1984) enzymes. However, in contrast to the behavior shown for these enzymes, the enzyme from the thermophilic bacterium PS3 (TF_1) when exposed to substoichiometric amounts of [α,γ-^{32}P]ATP did not retain [^{32}P]P_i or [α-^{32}P]ADP at catalytic sites after the enzyme was centrifuged through a Sephadex column (Yohda & Yoshida, 1987). This suggested that the TF_1 might not follow a sequential site mechanism. Such observations were important because in the binding change mechanism for ATP synthesis a similar sequential participation of catalytic sites is postulated. The mechanism is proposed to be general for all ATP synthases, and thus the result with TF_1 raised doubts about its validity.

Other considerations suggested that further exploration of the behavior of TF_1 might provide some clarification. In order for the release of products to be markedly promoted by ATP binding at an alternate site, it is not essential that the products be retained sufficiently long to pass through a Sephadex column. Retention for a time considerably longer than the turnover time when excess substrate was present would suffice. Another important characteristic demonstrated for the mitochondrial (Hutton & Boyer, 1979), chloroplast (Kohlbrenner & Boyer, 1983), and *E. coli* enzymes (Wood et al., 1987) is that when medium ATP concentration is lowered the bound ATP continues to undergo reversible interconversions to bound ADP and P_i. This behavior results in an increase in the number of water oxygens incorporated into each P_i formed. It thus seemed appropriate to use this approach to check whether, during hydrolysis of lowered concentrations of ATP by TF_1, ATP, ADP, and P_i might be transitorily tightly bound

† Supported by U.S. Public Health Service Grant GM11904.
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[1] Abbreviations: TF_1, CF_1, and MF_1, soluble ATPase portion of the ATP synthase from thermophilic bacterium PS3, chloroplasts, and mitochondria, respectively; P_i, inorganic phosphate; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/89/0428-6949$01.50/0

sufficiently long for there to be increased water oxygen incorporation into the P_i formed, and thus if TF_1 conforms to the sequential site behavior shown for the other F_1 ATPases. The results reported here show that this is indeed the case for nearly all the P_i formed from ATP by TF_1. A small amount of the product P_i arises from another catalytic pathway attributable to a weakly active enzyme form(s).

Experimental Procedures

Materials. ADP (dimonocyclohexylammonium salt) and pyruvate kinase from rabbit muscle were obtained from Sigma, and pyruvate kinase from *Bacillus stearothermophilus* was from Seikagaku Kogyo Co. (Japan). [^{18}O]Phosphoenolpyruvate was prepared as described previously (Stempel & Boyer, 1986). TF_1 was isolated from PS3 bacterium according to Yoshida et al. (1975) and stored in lyophilized form at −20 °C. Prior to use, the enzyme was dissolved in the appropriate experimental buffer. TF_1 contained 0.13 mol of ADP and less than 0.01 mol of ATP per mole of enzyme. Adenylate kinase activity of the TF_1 preparation was not detectable, less than 0.0003% of the ATPase activity.

^{18}O Exchange Conditions. ^{18}O exchange reactions were run in duplicate at pH 8.6 and at three temperatures: 60, 45, and 25 °C. A buffer containing 0.05 M Tris-HCl, 0.05 M KCl, and 3 mM $MgCl_2$ was used for experiments at temperatures below 60 °C, and 0.05 M glycine–KOH, 0.05 M KCl, and 3 mM $MgCl_2$ was used at 60 °C. The pH of the buffers was adjusted at 25 °C to a value that resulted in the designated pH at the working temperature. The indicated concentrations of [γ-^{18}O]ATP were generated from added ADP and [^{18}O]-phosphoenolpyruvate by the action of pyruvate kinase. Pyruvate kinase from rabbit muscle was used at 25 and 45 °C and from *B. stearothermophilus* at 60 °C; the concentration was 50 μg/mL when the ATP concentration was higher than 100 μM, and 100 μg/mL at lower ATP concentrations. The [^{18}O]phosphoenolpyruvate concentration was 3 mM when 1 or 2 mM ATP was used, 1 mM when 0.5 or 0.1 mM ATP was used, and 0.5 mM at lower ATP concentrations. The reaction mixture was incubated for at least 10 min prior to addition of the TF_1. This allowed complete conversion of added ADP to ATP. Then the samples were incubated for 15–30 min for enzymic hydrolysis. The concentration of TF_1 ranged from 1 μM when 1 μM ATP was used at 25 °C to much lower concentrations with higher ATP concentrations or at higher temperatures. The total volume was 2 mL with concentrations of ATP lower than 0.5 mM and 1.0 mL at higher ATP concentrations. Highly enriched [^{18}O]P_i (100 nmol) was added to the samples just before the reaction was quenched with an equal volume of chloroform. Control samples were run under the same conditions, but TF_1 was added after the chloroform quench. Dilution of the $P^{18}O_4$ isotopomer[2] gave a measure of the P_i present in the control samples and the P_i formed in samples by ATPase.

Analysis of ^{18}O Exchange Data. Purification of the phosphate and derivatization and analysis by gas chromatography/mass spectrometry were done according to Stempel and Boyer (1986). The distributions of the [^{18}O]P_i isotopomers in the control samples were subtracted from the samples where enzymic hydrolysis took place. The amounts of P_i in control samples ranged from less than 3% to about 20% (at the lowest ATP concentrations used) of that produced by ATPase activity. Calculations were made as described earlier (Hackney, 1980; Xue et al., 1988). The final distributions were normalized to those expected if the ATP used in the reaction had been 100% in ^{18}O.[3] Such normalization facilitates comparison of different experiments and allows ready assessment of the extent of oxygen exchange irrespective of different initial ^{18}O enrichments of ATP.

Catalytic pathways giving rise to phosphate oxygen exchanges are conveniently characterized by their partition coefficient, P_c, which is the probability that an oxygen of bound P_i formed from ATP will undergo exchange prior to release; the P_c varies from 0 for no oxygen exchange to 1 for complete oxygen exchange. If only one catalytic pathway is involved, the distributions of isotopomers can be accounted for by a single P_c. Comparison of the fits of experimental distributions with predicted distributions using a computer program has allowed detection of up to three pathways with different P_c values in modified CF_1 (Xue et al., 1988). In the present study, the same kind of comparisons were made.

To assess how well the observed and predicted distributions agreed, it was essential to know the overall precision of the analyses for ^{18}O-isotopomers. The differences from the means of duplicate measurements of the percentage amounts of the four isotopomers were summed. This was done for 17 pairs of enzyme incubations, each carried separately through the entire isolation and analysis procedures. The average of the sum of the differences ± SD was designated as the precision of duplicates and was equal to 0.89 ± 0.39% (n = 17). This good precision of analysis allows clear discrimination of whether observed distributions fit the theoretical distributions for one or two catalytic pathways.

Results

Distribution of [^{18}O]P_i Isotopomers Formed by Hydrolysis of [γ-^{18}O]ATP by TF_1 at 45 °C. For these and other similar experiments, a constant level of [γ-^{18}O]ATP was maintained during the enzymic reaction by use of highly enriched [^{18}O]phosphoenolpyruvate in the presence of pyruvate kinase and ADP. The distribution of [^{18}O]P_i isotopomers formed from the phosphoryl group of phosphoenolpyruvate was measured after acid hydrolysis in the presence of $HgCl_2$ (Stempel & Boyer, 1986).

The means of duplicates of the observed distributions of the four ^{18}O-isotopomers in the product P_i were compared to the theoretical distributions that gave the best fit for either one or two P_c values. For each of the four isotopomers, the difference between the observed and predicted values was calculated, and the sum of the four numbers was designated as the Δ%. The Δ% was calculated for each ATP concentration used and averaged for each experiment. Comparison of the average Δ% with the precision of duplicate analyses gave a measure of how well the observed distributions matched those expected for one or two catalytic pathways. This comparison showed that a single P_c value ($\Delta\%_{av}$ 12.80 ± 11.39, n = 9) could not account for the results. However, the predicted distributions for two P_c values agree with the observed results within experimental error ($\Delta\%_{av}$ = 0.57 ± 0.67, n = 9). The observed specific activities and the distributions of [^{18}O]P_i isotopomers formed at different concentrations of ATP are presented in Table I. In order to illustrate the comparison of observed and theoretical values, a bar graph of the predicated and observed distributions at 5 μM ATP is shown in Figure 1. This concentration of ATP was chosen for Figure

[2] The ^{18}O isotopomers of P_i containing 0–4 ^{18}O atoms are designated as $P^{18}O_0$, $P^{18}O_1$, $P^{18}O_2$, $P^{18}O_3$, and $P^{18}O_4$, respectively.

[3] A computer program for this calculation was kindly provided by Prof. David Hackney (Carnegie–Mellon University, Pittsburgh, PA).

Table I: Specific Activities and $[^{18}O]P_i$ Isotopomers Formed at Different Concentrations of ATP at 45 °C

						P_c values[b] and portion of P_i produced			
		% of $[^{18}O]P_i$ isotopomers				P_{c1}		P_{c2}	
[ATP] (μM)	sp act.[a]	$P^{18}O_0$	$P^{18}O_1$	$P^{18}O_2$	$P^{18}O_3$	value	% of P_i	value	% of P_i
2000	7.16	4.9	2.4	11.5	81.2	0.16	92.1	0.93	7.9
500	4.40	4.1	2.1	13.3	80.5	0.19	94.8	0.96	5.2
50	0.67	2.6	4.4	20.2	72.8	0.31	97.2	0.98	2.8
20	0.28	4.0	8.2	26.8	61.0	0.45	97.9	1.00	2.1
10	0.12	6.7	13.3	29.2	50.9	0.55	97.6	1.00	2.4
5	0.05	13.0	17.9	28.2	41.0	0.64	94.7	1.00	5.3
2.5	0.024	24.0	19.1	24.9	32.1	0.68	79.4	0.96	20.6
1.0	0.008	45.5	18.8	11.4	24.2	0.91	85.8	0.00	14.2
0.5	0.003	53.8	8.7	5.8	31.8	0.96	71.1	0.03	28.9

[a] Specific activity is measured as micromoles of P_i per minute per milligram. [b] Values that give the best agreement with the experimental data at the indicated ATP concentrations.

Table II: Specific Activities and $[^{18}O]P_i$ Isotopomers Formed at Different Concentrations of ATP at 60 °C

						P_c values[b] and portion of P_i produced			
		$[^{18}O]P_i$ isotopomers				P_{c1}		P_{c2}	
[ATP] (μM)	sp act.[a]	$P^{18}O_0$	$P^{18}O_1$	$P^{18}O_2$	$P^{18}O_3$	value	% of P_i	value	% of P_i
2000	17.82	5.2	1.1	5.3	88.3	0.07	92.8	0.96	7.2
1000	8.64	5.0	1.1	6.4	87.5	0.09	93.2	0.96	6.8
500	4.58	9.5	0.4	6.6	83.5	0.10	90.5	1.00	9.5
100	1.24	16.3	2.8	17.4	63.6	0.30	84.1	1.00	15.9
50	0.82	15.6	7.0	23.7	53.8	0.44	86.0	1.00	14.0
20	0.34	26.6	13.8	23.2	36.4	0.58	72.2	0.97	27.8
10	0.10	41.9	12.5	19.0	26.6	0.64	60.8	0.99	39.2
5	0.04	59.1	10.6	16.9	13.4	0.76	50.3	1.00	49.7

[a] Specific activity is measured as micromoles of P_i per minute per milligram. [b] Values that give the best agreement with the experimental data at the indicated ATP concentrations.

1 because of $\Delta\%$ values (8.09 and 1.03 for one P_c and two P_c values, respectively) were close to the $\Delta\%_{av}$ for data at other ATP concentrations. The important point shown by the results given in Table I and Figure 1 is that ATP hydrolysis occurs by one major catalytic pathway and that the P_c of this pathway increases markedly as the ATP concentration is decreased.

That extensive oxygen exchange occurs with hydrolysis by TF_1 is shown by the appearance of considerable $P^{18}O_0$, $P^{18}O_1$, and $P^{18}O_2$ isotopomers; since these are normalized distributions, the sample would be 100% $P^{18}O_3$ if no exchange had occurred. From Table I, it is evident that at 5 μM ATP or above over 90% of the P_i was produced by one major pathway. The P_c for this major pathway increases from 0.16 to 0.96; a detectable ATP modulation of the P_c also occurs for the minor pathway which increases to a P_c of close to 1.0 (indicating a high extent of oxygen exchange) as the ATP concentration decreases from 2000 to 10 μM. As P_c values approach 1.0, the average number of reaction reversals before P_i is released changes markedly with very small changes in P_c, and, thus, small differences in relatively high P_c values are experimentally readily distinguishable. This gives confidence that the appearance of a P_c value close to 1.0 means that some P_i is being formed by a pathway with very extensive oxygen exchange.

The possibility that the P_i not enriched with ^{18}O was produced from the unenriched ADP present by adenylate kinase action needed consideration. The ADP could give rise to unenriched ATP which would then be hydrolyzed by the ATPase. However, this seemed quite unlikely because of the lack of detectable adenylate kinase activity in the TF_1. In addition, the amount of unenriched P_i produced considerably exceeded the amount of unenriched ADP present.

At ATP concentrations of 1 and 0.5 μM, it was not possible to distinguish between the P_c values for the major portion of P_i produced and the high-exchange component which was present at all higher ATP concentrations. At these low medium ATP concentrations, it was also possible to detect some P_i formation with a P_c close to 0. This might represent some minor contaminating enzymic activity capable of hydrolyzing ATP with practically no exchange. The concentrations of TF_1 used at the low ATP concentrations were more than 100 times those used at the highest ATP concentrations to compensate for the reduced ATPase activity.

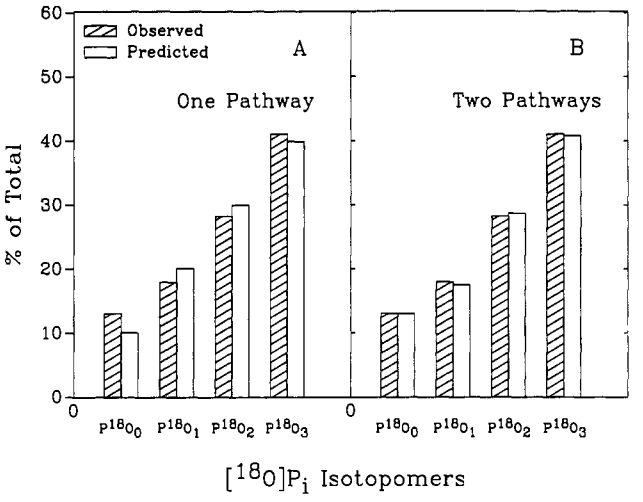

FIGURE 1: Comparison of the distributions of $[^{18}O]P_i$ isotopomers formed by the hydrolysis of 5 μM [γ-^{18}O]ATP by TF_1 at 45 °C with the predicted distributions for hydrolysis with one P_c (panel A) or two P_c values (panel B).

Distribution of $[^{18}O]P_i$ Isotopomers Formed by Hydrolysis of [γ-^{18}O]ATP by TF_1 at 60 °C. Hydrolysis of ATP at 60 °C was performed in essentially the same way as at 45 °C with the following minor differences. Thermostable pyruvate kinase was used to maintain the ATP concentration constant, and glycine–KOH buffer was used because Tris buffer changes the pH markedly with temperture. Data on the distributions

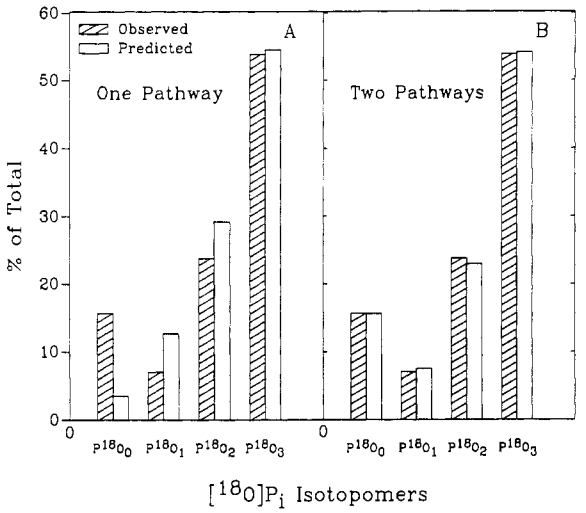

FIGURE 2: Comparison of the distribution of $[^{18}O]P_i$ isotopomers formed by the hydrolysis of 50 μM [γ-^{18}O]ATP by TF_1 at 60 °C with the predicted distributions for hydrolysis with one P_c (panel A) or two P_c values (panel B).

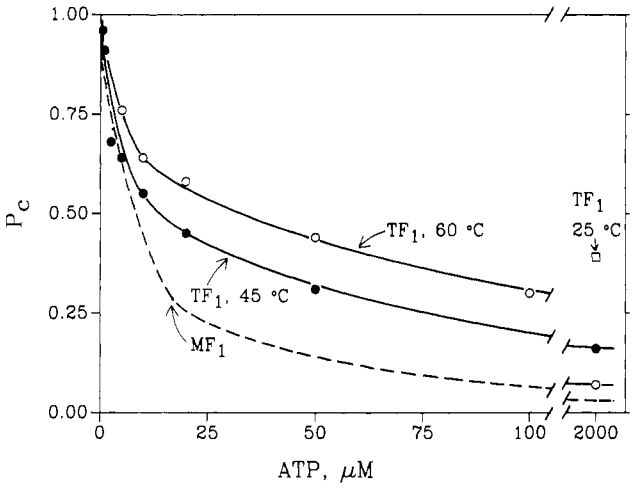

FIGURE 3: Dependence of P_c on ATP concentration during ATP hydrolysis by TF_1 at 45 °C (●) and 60 °C (○). The analogous curve (dashed line) is from the data of O'Neal and Boyer (1984) for MF_1.

of the $[^{18}O]P_i$ isotopomers formed are presented in Table II. The predicted distributions for one catalytic pathway with one P_c value matched the observed ones very poorly at all ATP concentrations ($\Delta\%_{av}$ = 21.18 ± 6.83, n = 7). Using predicted distributions for two P_c values improved the matching remarkably ($\Delta\%_{av}$ = 0.38 ± 0.6, n = 7). Data for 5 μM ATP are not included in the calculation of $\Delta\%_{av}$ for matching with two P_c values because the observed $\Delta\%$ (6.31) was much bigger than for other ATP concentrations, and the small amount of P_i formed made the results at 5 μM of questionable reliability.

The observed distribution at 50 μM ATP is compared with the predicted distributions for one P_c and two P_c values ($\Delta\%$ 24.10 and 1.48, respectively) in Figure 2. Figure 3 shows the change in P_c of the major pathway with ATP concentration at 45 and 60 °C. At 2 mM ATP, the P_c representing the major pathway at 45 °C was higher than at 60 °C. The distribution of $[^{18}O]P_i$ isotopomers during hydrolysis of 2 mM ATP at 25 °C gave an even higher P_c value (0.39, Figure 3) than at 45 °C (0.16). At low ATP concentrations, the opposite is true; P_c values are lower for the major pathway at 45 °C than at 60 °C. It is also clear from the data in Table II that the elevated temperature not only increased modulation of the intermediate exchange but also increased the portion of P_i formed by the catalytic pathway characterized by very high exchange.

Discussion

In the binding change mechanism for ATP synthesis, the sequential participation of catalytic sites results in retention of tightly bound ATP at a catalytic site until ADP and P_i bind at another catalytic site and promote the conformational changes that allow use of the protonmotive force for the binding changes leading to release of ATP. The important prediction that at low ADP or P_i concentrations during net synthesis a tightly bound ATP should be retained at catalytic sites was shown for the chloroplast enzyme by Rosen et al. (1979) and for the mitochondrial enzyme by Gresser et al. (1979). In addition, as demonstrated by the extent of water oxygen incorporation into the ATP formed, bound ATP, ADP, and P_i continue to undergo interconversion until conditions for ATP release are attained (Hackney et al., 1979; Stroop & Boyer, 1985). These important observations for ATP synthesis suggest but do not require that a similar sequential site behavior would be shown for the hydrolysis of ATP by F_1 ATPases separated from the F_0 component. That this has proved to be the case has been rewarding as it means that the characteristics of the isolated F_1 can be taken as reflecting catalytic properties of the biologically more important reaction, the formation of ATP by the synthase. It was such considerations that made it important to examine the interesting behavior of the TF_1 observed by Yohda and Yoshida (1987).

The results reported here demonstrate that, for the major catalytic pathway of ATP hydrolysis by TF_1, as ATP concentration is lowered, bound ATP, ADP, and P_i are retained at a catalytic site and continue to undergo reversible interconversion. The number of reversals of bound ATP hydrolysis before P_i is released increases sharply when the ATP concentration is lowered into the micromolar range. The increase to a P_c above 0.95 (Figure 3, 45 °C) as the ATP concentration is lowered corresponds to the occurrence of at least 19 reversals of bound ATP hydrolysis to bound ADP and P_i before the P_i is released. The P_c of about 0.16 at high ATP concentrations corresponds to only 0.19 reversal. Thus, the relative rate of escape of bound P_i from the exchange reaction has been increased by at least 100-fold. Such an increase clearly harmonizes with the need for participation of sequential sites on the enzyme to attain significant reaction rates. The modulation by ATP is greater at 60 °C than at 45 °C; the P_c at 60 °C starts at a lower value than at 45 °C at high ATP concentrations and has a higher value than that at 45 °C at low ATP concentrations. This might be a reflection that 60 °C is closer to the normal operating temperature of the enzyme in vitro. Also, the enzyme has been found to have two temperature optima, at 20 and 70 °C, with a minimum at 45 °C (Yoshida et al., 1975), and thus different properties may not be unexpected at the two temperatures.

The maximum rate of net ATP hydrolysis at 60 °C results in cleavage of one ATP per enzyme molecule every 8.9 ms. The rate of reaction reversal when P_i is not released at low medium ATP concentration cannot be calculated from the present data. For illustrative purposes, however, it may be assumed that a reaction reversal prior to binding of an ATP at an alternate site requires the same amount of time as does the net hydrolysis of one ATP per enzyme molecule. If this were so, for a P_c of 0.95, the ADP and P_i would need to be retained at the catalytic site for about 1.5 s before P_i release. Such a rate would obviously allow a sufficiently rapid release of products so that little or no bound ADP or P_i would be found with the enzyme after centrifugation through a Sephadex

column. Thus, these oxygen exchange results and the similar behavior for other enzymes mentioned earlier are consistent with the view that the binding change mechanism is general for the F_1 ATPases.

A sensitive and useful feature of the ^{18}O methodology is that it can reveal the presence of more than one catalytic pathway. For the mitochondrial (Hutton & Boyer, 1979) and chloroplast (Kohlbrenner & Boyer, 1983) enzymes, within experimental error, all the product P_i arises by one catalytic pathway at all enzyme concentrations. However, when one catalytic site on the CF_1 is blocked by covalent labeling with 2-azido-ATP, the two remaining catalytic sites cleave ATP by two pathways characterized by P_c values different from the intact enzyme (Melese et al., 1988). These prior results, and the reliability of the approach as covered under Experimental Procedures and Results, give confidence that some ATP is being hydrolyzed by the TF_1 preparation by a second pathway characterized by a high P_c, that is, with many reversals of bound ATP hydrolysis before P_i release.

Because this secondary pathway has a higher P_c value, it is likely that the enzyme form involved has a slow turnover relative to the more active enzyme present. This would mean that the portion of the total enzyme involved in the pathway could be considerably higher than the fraction of the total P_i released by the pathway. In studies of the release of [^{3}H]ADP from TF_1 when MgATP is added, Yoshida noted that a considerable fraction of the [^{3}H]ADP was not released by the time the enzyme attained maximum activity (M. Yoshida, unpublished results). The results reported here offer a plausible explanation for this behavior. It seems likely that the slowly released [^{3}H]ADP was on a less active enzyme form responsible for the secondary catalytic pathway.

As demonstrated by Yoshida and Allison (1986), the TF_1 when exposed to ADP or MgADP will retain about one tightly bound ADP after centrifugation through a Sephadex column. Although most of this ADP is at a catalytic site on the β subunit, the kinetic behavior of the TF_1 with one tightly bound ADP suggests that it may not represent a normal intermediate in the catalysis. As observed with the mitochondrial F_1 (Hackney, 1979) and chloroplast F_1 (Feldman & Boyer, 1985), as well as with TF_1 (Yoshida & Allison, 1986), such F_1 forms with tightly bound ADP but no bound P_i at a catalytic site, after exposure to Mg^{2+}, show a delay in the attainment of a maximum catalytic rate after addition of MgATP. In the resulting cooperative steady-state catalysis, tightly bound ADP and P_i are present on a transient intermediate form that is quickly converted to a form that binds bound ADP and P_i loosely. The products readily dissociate from this latter form.

The fraction of P_i formed by the secondary pathway at 60 °C increases markedly as the ATP concentration is lowered. Such a result would be anticipated if the apparent K_m value for the less active enzyme were considerably lower than for the normal enzyme. The small increase in the fraction of P_i produced by the secondary pathway at 60 °C as the ATP concentration is increased from 50 to 2000 μM might be the result of some remaining catalytic cooperativity between sites with a high apparent K_m for the enzyme involved.

An enzyme form with a low K_m and high P_c could arise if its structural alterations were such as to decrease the cooperativity between catalytic sites. Indeed, mutant enzyme forms with low catalytic activity have been observed to retain the ability to do slow single-site catalysis with quite high affintiy for ATP and to show extensive reaction reversal before P_i is released (Noumi et al., 1986; Duncan & Senior, 1985; Wood et al., 1987). It is also possible that the secondary pathway could arise from partially dissociated enzyme or separated β subunits.

In the binding change mechanism, all ATP that binds at any of the three catalytic sites on the F_1 ATPases undergoes subsequent hydrolysis in an equivalent manner. An alternate suggestion that is sometimes made is that the site that binds ATP most tightly, as in single-site catalysis, contributes little or nothing to the steady-state catalysis with excess ATP present. However, recent contributions (Penefsky, 1985; Cunningham & Cross, 1988) give convincing evidence that the high-affinity site of MF_1 participates in turnover at higher ATP concentrations. Such participation is also in accord with the findings of Yohda and Yoshida (1987) with TF_1. In addition, Yohda and Yoshida showed that the dependency of the initial velocity on ATP concentration is compatible with the binding of ATP at a second and third site, resulting in an increase in the rate of catalysis. All present data appear to be consistent with the equivalent participation of three sites in catalysis, and such participation is appealing as it can allow for equivalent structural interactions among the various subunits. However, it must be noted that the possibility that only the tight site and one other catalytic site participate cooperatively, and that a third catalytic site has some type of control function, has not been conclusively eliminated.

ACKNOWLEDGMENTS

The excellent technical assistance of Kerstin Stempel is gratefully appreciated.

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S-Band EPR Studies of the S_2-State Multiline Signal from the Photosynthetic Oxygen-Evolving Complex†

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Received January 17, 1989; Revised Manuscript Received May 4, 1989

ABSTRACT: The S_2-state multiline EPR signal observed in photosynthetic membrane preparations has been previously well characterized at X-band frequencies (9.1-9.5 GHz). These studies have indicated that the signal, centered at $g = 2$, arises from a multinuclear mixed-valence Mn center of the O_2-evolving complex. In the present study, the multiline EPR signal from spinach photosystem II enriched membranes is characterized at an S-band frequency (3.9 GHz). At this lower frequency, the resolution and complexity of the signal increase markedly compared with its appearance in the X-band. While the multiline signal covers similar magnetic field ranges at the two frequencies, the S-band signal has a greater number of lines, narrower line widths, and a different overall appearance. Replacement of Cl^- with Br^- or ^1H_2O with ^2H_2O in the buffer shows that neither exchangeable Cl^- nor protons cause superhyperfine structure in the S-band multiline signal. Membrane preparations oriented on mylar sheets show dependence of the S-band signal on the angle between the mylar sheet normal and the magnetic field direction, indicating that the multiplicity of lines is in part due to signal anisotropy. The results, combined with previous work at X-band, indicate that a minimal working model for the species responsible for the multiline signal is a mixed-valence binuclear Mn complex with an anisotropic hyperfine interaction that includes second-order contributions.

The multiline EPR signal, centered at $g = 2$, is observed in photosynthetic membrane preparations in the S_2-state, one of several oxidative intermediates (S_0 through S_4) of the O_2-evolving system. Following its discovery (Dismukes & Siderer, 1981), another S_2-state signal at $g = 4.1$ was soon found (Casey & Sauer, 1984; Zimmermann & Rutherford, 1984). These two remain the only known EPR signals that have been positively identified with any of the S-states of the O_2-evolving complex. Due to the efforts undertaken in several laboratories, the multiline signal has been well characterized at X-band frequencies (Dismukes et al., 1982; Hansson et al., 1987; de Paula et al., 1987; Zimmermann & Rutherford, 1986). However, despite these extensive studies, our understanding of the origin of this signal, which is necessary for an understanding of the chemical mechanism of O_2 evolution, is not complete.

The X-band multiline signal covers a span of about 150 mT and consists of 16-20 main lines that are approximately evenly spaced and have numerous small peaks and shoulders. Models that explain the signal's appearance as arising from monomeric Mn with a small zero-field splitting have been ruled out on the basis of a lack of large-scale signal anisotropy (Hansson et al., 1984; Rutherford, 1985). Instead, the signal is thought to arise from a mixed-valence multinuclear Mn complex. Models for bi-, tri-, and tetranuclear Mn clusters have been proposed (Dismukes et al., 1982; Andréasson et al., 1983; Hansson et al., 1987; de Paula et al., 1987).

In order to understand the source of the many small peaks and shoulders of the multiline signal, investigations have been made into the influence of possible exchangeable ligands to Mn. These include the substrate water and the anion Cl^-, which is required for O_2 evolution (Critchley, 1985). Substitution of water ^{16}O with the magnetic ^{17}O nucleus produced broadening of details of the signal (Hansson et al., 1986; Andréasson et al., 1988). This result implies that oxygen from water becomes closely associated with the Mn center by the time it is in the S_2-state. Similar investigations using deuterated water have been less clear. While no effect of ^2H_2O was found by one laboratory (Yachandra et al., 1986), an increase in the resolution of the multiline signal's small peaks and shoulders was found by another (Nugent, 1987). In the case of Cl^-, substitution experiments using Br^- as the substituting ion revealed no change in the multiline signal from spinach photosystem II (PSII)[1] preparations (Yachandra et al., 1986). Thus, although H_2O may have subtle effects on the line width of the multiline signal, neither H_2O nor exchangeable Cl^- has been found to be responsible for the small

† This work was supported by grants from the Swedish Natural Science Research Council and the Axel and Margaret Ax:son Johnson Foundation. A.H. was supported by a postdoctoral fellowship from the American-Scandinavian Foundation during 1988.

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[1] Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; OEC, oxygen-evolving complex; PPBQ, phenyl-*p*-benzoquinone; PSII, photosystem II.

0006-2960/89/0428-6954$01.50/0