Kinetics of ATP Hydrolysis by F_1-ATPase and the Effects of Anion Activation, Removal of Tightly Bound Nucleotides, and Partial Inhibition of the ATPase by Covalent Modification[†]

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ABSTRACT: Eadie–Hofstee plots (v/[S] vs. v) of the kinetics of ATP hydrolysis by purified bovine heart mitochondrial F_1-ATPase (MF_1) over a substrate (MgATP) concentration range of 1–5000 μM were curvilinear, indicating negative cooperativity with respect to [MgATP] as originally shown by Ebel & Lardy (1975) [Ebel, R. E., & Lardy, H. A. (1975) *J. Biol. Chem. 250*, 191–196]. The data were computer analyzed for the best fit of the least number of straight lines, each representing a different apparent K_m and V_{max}. The best fits for MF_1 and TF_1 from the thermophilic bacterium PS3 were three lines in each case. The upper limits of the apparent K_m values for MF_1 were of the order of 10^{-6}, 10^{-4}, and 10^{-3} M, and the corresponding apparent V_{max} values (per minute per milligram of protein) were in the range of micromoles or less for the lowest K_m line and decamicromoles for the other two. The results for TF_1 were very similar. The presence of an activating anion (10 mM $KHCO_3$) in the MF_1 assay medium increased the overall V_{max} by about 50% and eliminated the high K_m but had essentially no effect on the intermediate and low K_m's, indicating retention of negative cooperativity in the corresponding substrate concentration range. Kinetic data for MgITP as substrate also yielded two K_m values (in the absence of $KHCO_3$) differing by about 10^4-fold. The relationship between [^{14}C]dicyclohexylcarbodiimide ([^{14}C]-DCCD) binding to MF_1 and activity inhibition was linear up to approximately 1 mol of DCCD bound/mol of MF_1. At this point, the degree of inhibition was about 95%. Further [^{14}C]DCCD binding up to and beyond 2 mol/mol of MF_1 inhibited the remaining 5% activity in small increments up to about 99%. [^{14}C]DCCD binding occurred exclusively on the β subunits. Only at $\geq$2 mol of DCCD/mol of MF_1 was a marginal labeling of the γ subunit detected. Eadie–Hofstee plots of ATP hydrolysis by 95% inhibited MF_1 containing $\geq$1 mol of [^{14}C]DCCD/mol of enzyme were biphasic. The highest K_m and associated V_{max} values were eliminated, while the intermediate and low K_m values were close to those of the unmodified MF_1. These results agree with the apparent inactivation by DCCD of one β subunit (out of three) per MF_1 molecule. The relevance of the above analyses to data for unisite, bisite, and trisite ATP hydrolysis by MF_1 as reported recently [Grubmeyer, C., Cross, R. L., & Penefsky, H. S. (1982) *J. Biol. Chem. 257*, 12092–12100; Cross, R. L., Grubmeyer, C., & Penefsky, H. S. (1982) *J. Biol. Chem. 257*, 12101–12105] has been discussed.

It is generally accepted that the five unlike subunits of the mammalian mitochondrial F_1-ATPase are present in the enzyme molecule in the molar ratio $\alpha_3\beta_3\gamma\delta\epsilon$ (Esch & Allison, 1979; Senior & Wise, 1983), and various lines of evidence have indicated that the β subunits alone (Budker et al., 1977; Esch & Allison, 1978; Lauquin et al., 1980) or the α and β subunits together (Williams & Coleman, 1982; Senior & Wise, 1983) comprise the active sites of the enzyme. Thus, each F_1-ATPase molecule is considered to contain three active sites, and recent evidence has indicated that the three individual active sites on each soluble F_1-ATPase isolated from beef heart mitochondria (F_1)[1] molecule are catalytically active for ATP hydrolysis (Grubmeyer et al., 1982; Cross et al., 1982). These sites have also been shown to interact during ATP hydrolysis in a manner which results in (a) negative cooperativity with respect to [ATP] (Schuster et al., 1975) and (b) positive catalytic cooperativity such that ATP binding to the second and third sites greatly facilitates turnover at the first site (Grubmeyer et al., 1982; Cross et al., 1982). In the latter studies, Grubmeyer et al. (1982) used an ATP:F_1 molar ratio of 1:3 in order to study unisite catalysis and obtained a turnover rate of 1×10^{-4} s^{-1} as V_{max} for unisite ATP hydrolysis by F_1. Cross et al. (1982) derived K_m and V_{max} values for bisite and trisite ATP hydrolysis by F_1 from double-reciprocal Lineweaver–Burk plots and arrived at the conclusion that trisite turnover (V_{max} = 600 s^{-1}) is increased 10^6-fold as compared to unisite turnover, primarily because substrate binding to the second and third sites facilitates product (ADP) release from the first site by the same factor.

In the present study, kinetic data for ATP hydrolysis by purified mitochondrial F_1 were obtained under several different conditions, using a wide range of substrate concentrations, from 1 μM to 5 mM. The results were graphed as Eadie–Hofstee plots (v/[S] vs. v) and computer analyzed for the best fit of the least number of slopes. The following different conditions were used: (a) F_1 as isolated by the procedure of Senior & Brooks (1970) without the heat-activation step; (b) F_1 activated by incubation with ATP and then freed of added ATP and products by gel filtration; (c) F_1 treated according to Garrett & Penefsky (1975) to remove tightly bound nucleotides; (d) F_1 of (a–c) assayed in the presence of 10 mM HCO_3^- which is knwon to stimulate ATP hydrolysis and eliminate negative cooperativity as discerned by double-reciprocal plots (Ebel & Lardy, 1975); (e) F_1 in the presence of ITP as substrate; (f) F_1 treated with [^{14}C]DCCD to the extent that $\geq$1.0 mol of [^{14}C]DCCD was bound per mol of F_1 on the β subunits.

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[1] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; F_1 (or MF_1) and TF_1, soluble F_1-ATPase isolated from beef heart mitochondria and the thermophilic bacterium PS3, respectively; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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In addition, the kinetics of ATP hydrolysis in the absence and presence of HCO_3^- were analyzed for the ATPase from thermophilic bacterium PS3 (TF_1), which lacks tightly bound nucleotides.

Materials and Methods

ATPase Assay. F_1-ATPase was prepared from bovine heart mitochondria according to Senior & Brooks (1970), except that the heat-activation step was omitted. Before use, the enzyme preparation was filtered through centrifuge–Sephadex G-50 (fine) columns (Penefsky, 1979) to desalt. This procedure appears also to remove most or all of the nucleotides from F_1, except those bound to the noncatalytic sites (Grubmeyer et al., 1982). ATPase activity was measured by the ATP regenerating system as before (Stiggall et al., 1979). All the reagents used in the ATP regenerating system were examined by the luciferin–luciferase assay (Lundin et al., 1976) at severalfold the concentration used in the ATPase assay for possible ATP contamination. In addition, the ATPase assay was conducted in the absence of added ATP and the presence of severalfold concentrations of other additives, including F_1, to assess the presence of contaminating ATP or ADP in the reagents. Both tests failed to detect any ATP contamination in the assay reagents, even though in the luciferin–luciferase assay 10 nM ATP was easily detectable. When used in the ATP regenerating assay in the absence of added ATP, 20-fold the highest F_1 concentration used in the experiments reported here resulted in a marginal A_{340} decrease, suggesting the possible presence of small amounts of ADP in F_1 preparations. This marginal level decreased further when F_1 was passed through two consecutive centrifuge–Sephadex columns. However, at the normal levels of F_1 used in our experiments, any ADP, if present, had essentially no effect on the measured rates. Protein was determined by the method of Lowry et al. (1951).

Removal of Tightly Bound Nucleotides. F_1-ATPase was depleted of tightly bound nucleotides according to Garrett & Penefsky (1975). The enzyme was filtered through a Sephadex G-50 (medium) column equilibrated and eluted with 100 mM Tris–sulfate, pH 8.0, containing 50% glycerol and 4 mM EDTA. The eluted enzyme was precipitated by addition of saturated ammonium sulfate to 0.6 saturation and collected by centrifugation. It was then dissolved in a small volume of the same buffer as above, saturated ammonium sulfate added to 0.6 saturation, and stored in the refrigerator. The A_{280}:A_{260} ratio was checked to estimate the extent of nucleotide depletion (Garrett & Penefsky, 1975). Preparations which appeared not adequately depleted of tightly bound nucleotides were filtered through Sephadex once again as above. Those used for kinetic studies had an A_{280}:A_{260} ratio ≥ 1.85.

Treatment of F_1 with [^{14}C]DCCD. F_1-ATPase (1 mg/mL) was incubated at 30 °C with 132 μM [^{14}C]DCCD ($\sim$75 000 cpm/nmol) in a mixture containing 40 mM MES, 32 mM Tris, 0.25 M sucrose, 2 mM EDTA, and 4 mM ATP, pH 6.8. At various intervals, aliquots were removed and filtered through two successive centrifuge–Sephadex G-50 (fine) columns equilibrated in 40 mM MES, 40 mM Tris, 1 mM K_2HPO_4, and 0.25 M sucrose, pH 8.0 (Penefsky, 1979). Then ATPase activity, F_1-bound radioactivity, and the amount of protein recovered were determined. In addition, samples treated as above were electrophoresed on 16% Laemmli sodium dodecyl sulfate–acrylamide gels (Laemmli, 1970), and total radioactivity on each gel was determined after staining and destaining.

Gel Electrophoresis. Distribution of label among the subunits of F_1 was determined by electrophoresis of F_1 as above on a 1.5-mm-thick slab gel, using the procedure of Laemmli as modified by Merle & Kadenback (1980). After the gels were stained with Coomassie blue and destained, an 8-mm strip was cut longitudinally, scanned at 580 nm in a Gilford gel scanner, then frozen on dry ice, and cut into 1-mm-thick slices. Each slice was digested in 0.5 mL of 30% H_2O_2 by heating at 60 °C overnight and dissolved in 5 mL of Beta Blend, and its radioactivity was measured in a Beckman LS-250 scintillation counter. For further determination of the distribution of [^{14}C]DCCD between the α and β subunits of F_1, the labeled enzyme was subjected to isoelectric focusing and autoradiography as follows. [^{14}C]DCCD-treated F_1-ATPase (100 μg) was passed through centrifuge–Sephadex columns equilibrated in water and was added to a mixture containing 0.5 mL of acrylamide/bis(acrylamide) (45%/0.6%), 1 mL of 10% Nonidet P-40, 0.25% ampholine (pH 3.5–10), 2.75 g of urea, and water to a final volume of 5 mL. After being degassed, polymerization was initiated by addition of 30 μL of 10% ammonium persulfate and 5 μL of N,N,N',N'-tetramethylethylenediamine. The mixture was then poured into a 0.15 × 3.5 × 8 cm slab between glass plates with Gel Bond film spread over the inside surface of one plate. After polymerization, the glass plates and spacers were removed, and the gel attached to the film was electrophoresed at 20 °C in a Bio-Rad flat-bed electrophoresis cell with 0.1 M NaOH (extensively degassed) at the cathode and 0.02 M H_3PO_4 at the anode. The electrophoresis schedule was (1) 100 V for 10 min, (2) 200 V for 15 min, (3) 300 V for 30 min, (4) 400 V for 30 min, and (5) 500 V for 5 h. After electrophoresis, the gel was fixed in 12.5% trichloroacetic acid, stained in an aqueous mixture of 25% 2-propanol, 10% acetic acid, and 0.05% Coomassie blue for 20 min, destained in 10% acetic acid, and dried in a forced-air oven at 60 °C. Autoradiography was carried out for 96 h at room temperature, using Kodak XAR-5 film.

The sources of materials used were as follows. Lactate dehydrogenase, pyruvate kinase, phosphoenolpyruvate, and luciferin were obtained from Sigma; ATP and luciferase were from Boehringer; NADH and ITP were from P-L Biochemicals; [^{14}C]DCCD was from Research Products International; acrylamide was from Baker; bis(acrylamide) was from Eastman Kodak; ampholine was from LKB; Nonidet was from Shell Chemical Co., London. TF_1 was the generous gift of Dr. M. Yoshida, Jichi Medical School, Japan.

Results

Analysis of the Kinetics of ATP Hydrolysis by F_1. The results of Grubmeyer et al. (1982) and Cross et al. (1982) have indicated that each of the three active sites on a molecule of F_1-ATPase is capable of ATP hydrolysis and that the overall activity of the enzyme is a consequence of the turnover of all three sites. These authors have published data for unisite, bisite, and trisite ATP hydrolysis by purified bovine heart F_1. For estimation of the rate of unisite ATP hydrolysis, they used a reaction mixture containing a 1:3 molar ratio of ATP:F_1 and obtained a rate designated as $V_{max} = 1 \times 10^{-4}$ s^{-1}. The data for bisite (i.e., two sites together) and trisite (i.e., three sites together) were derived from double-reciprocal Lineweaver–Burk plots at high ATP:F_1 ratios. We have employed a wide range of MgATP concentrations (1 μM–5 mM) at a constant level of F_1 and have plotted the kinetic data as $v/[S]$ vs. v (Eadie–Hofstee plot) where v is the initial reaction rate and [S] is the MgATP concentration in the presence of a constant additional amount of 1 mM $MgSO_4$ in the assay mixture. The results are shown in Figure 1 (open circles). Since Eadie–Hofstee plots of a unisite enzyme obeying simple Michaelis–Menten kinetics are linear over a noninhibitory range of

Table I: Kinetics of ATP Hydrolysis by F_1-ATPase and the Effects of Activation and Limited Chemical Modification[a]

kinetic constant (apparent)	no additions	$+HCO_3^-$	activated[b]	activated + HCO_3^-	+DCCD
$K_{m,1}$	$\leq 10^{-6}$	$\leq 10^{-6}$	$\leq 10^{-6}$	$\leq 10^{-6}$	$<10^{-6}$
$V_{max,1}$	≤ 4	≤ 2	≤ 10	≤ 8	≤ 0.2
$K_{m,2}$	1.6×10^{-4}	1.6×10^{-4}	1.9×10^{-4}	1.8×10^{-4}	1.4×10^{-4}
$V_{max,2}$	47	120	70	160	5
$K_{m,3}$	10^{-3}		10^{-3}		
$V_{max,3}$	29		34		

[a] V_{max} values are expressed as micromoles of ATP hydrolyzed per minute per milligram of protein, and K_m values are in molar units. [b] The Eadie–Hofstee plot in this case could also be analyzed to fit two slopes.

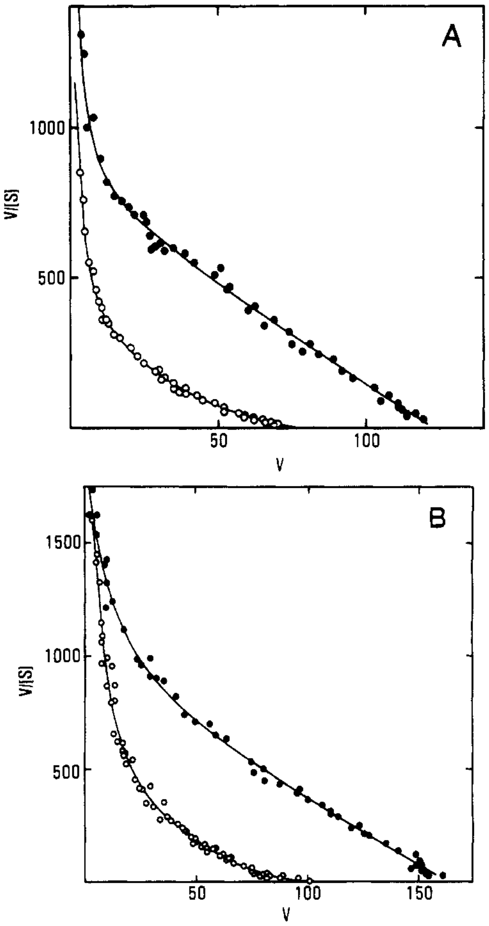

FIGURE 1: Eadie–Hofstee plots of the kinetics of ATP hydrolysis by F_1 (A) and activated F_1 (B) assayed in the absence (○) and presence (●) of 10 mM $KHCO_3$. The enzyme concentration was 0.5 μg/mL of the assay medium. All other conditions are described under Materials and Methods and under Results. The lines drawn through the experimetnal points in this and Figures 2 and 5 were computer derived from the calculated K_m and V_{max} values. Also in this and in Figures 2 and 5, V is in micromoles per minute per milligram of protein, and [S] for MgATP (or MgITP in Figure 2) is in millimolar.

substrate and product concentrations, the curvilinear data of Figure 1A were computer analyzed with the assumption that they were generated by a reaction mixture containing several discrete unisite ATPases, each having its own specific K_m and V_{max}. This curve-fitting exercise was attempted with the objective of finding the least number of lines of different slopes which when combined best accommodated the experimental points.[2] It was found that the data of Figure 1A (open circles) best fitted three lines, which agreed with the presence in F_1 of three active sites of inequivalent catalytic properties. The K_m and V_{max} (apparent)[3] values derived from the three lines are shown in column 2 of Table I. The data indicate three K_m values: a low K_m in the micromolar range, an intermediate K_m in the hundred micromolar range, and a high K_m in the thousand micromolar range. The V_{max} associated with the lowest K_m is the smallest, while the other two are in the same range and roughly 1 order of magnitude larger. It is important to point out that in these and other analyses shown in Table I, the $K_{m,2}$ and $V_{max,2}$ values are the most reliable numbers. The others are only rough estimates because substrate concentration could not be extended far below 1 μM and above 5 mM without risking the reliability of the kinetic data. At very low [ATP], reliable rates could still be obtained by increasing F_1 concentration. However, when [ATP] was low, at $[F_1]$ >0.8 μg/mL, the rates were no longer a linear function of enzyme concentration and began to decline. Accordingly, we have refrained in Table I from giving precise numbers where data limitation necessitated caution and have confined ourselves to reporting upper limits of order of magnitude. The above comments should be considered, therefore, in evaluating data shown in Table II as well.

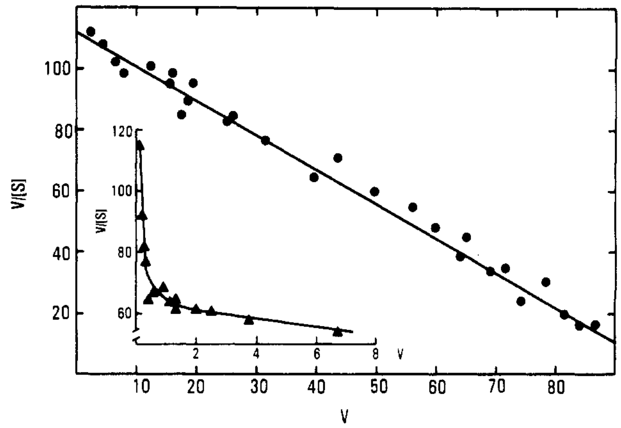

FIGURE 2: Eadie–Hofstee plots of the kinetics of ITP hydrolysis by activated F_1. The assay conditions were the same as those described under Materials and Methods for assay of ATP hydrolysis, except that in the large frame the ITP concentration was 20–5000 μM and the F_1 concentration was 0.7 μg/mL, and in the inset they were, respectively, 1.0–100 μM and 7 μg/mL. Activation of F_1 was carried out as described in the text.

Effect of Activation of F_1 by Preincubation with ATP or by Addition of Bicarbonate to the Assay Mixture. The open circles in Figure 1B show the kinetic data for the same F_1 preparation of Figure 1A (open circles) after the enzyme was incubated for 30 min at 30 °C with 4 mM ATP and 2 mM EDTA and passed through two consecutive Sephadex columns to remove the additives. This treatment increased the V_{max} to >100 μmol of ATP min^{-1} mg of F_1^{-1} which is comparable

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[2] Gresser et al. (1982) have used a different procedure for obtaining K_m and V_{max} values for a biphasic Eadie–Hofstee plot. Analyzing their data by our procedure, we have obtained essentially the same K_m and V_{max} values as reported by Gresser et al.

[3] Although not so indicated for simplicity, all K_m and V_{max} values given in this paper should be considered as apparent K_m and apparent V_{max} values for ATP hydrolysis at 30 °C by F_1 or TF_1.

Table II: Kinetics of ATP Hydrolysis by ATPases Lacking Tightly Bound Nucleotides[a]

kinetic constant (apparent)	MF_1	TF_1	$TF_1 + HCO_3^-$
$K_{m,1}$	$\leq 10^{-6}$	$\leq 10^{-5}$	$\leq 10^{-5}$
$V_{max,1}$	≤ 3	≥ 1	≥ 1
$K_{m,2}$	2.6×10^{-4}	3×10^{-4}	2.8×10^{-4}
$V_{max,2}$	103	9	16
$K_{m,3}$		10^{-3}	10^{-3}
$V_{max,3}$		15	18

[a] MF_1, bovine mitochondrial F_1; TF_1, thermophilic bacterium F_1. K_m values are in molar units, and V_{max} values are expressed as micromoles of ATP hydrolyzed per minute per milligram of protein.

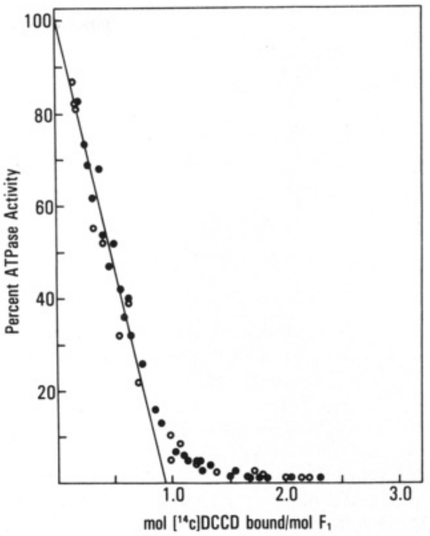

FIGURE 3: Correlation between ATPase activity inhibition and [^{14}C]DCCD binding to F_1. Experimental conditions have been described under Materials and Methods. (●) Radioactivity bound to F_1 after filtration through two successive Sephadex G-50 (fine) columns; (○) radioactivity bound to F_1 after Sephadex column filtration as above and electrophoresis on 16% Laemmli sodium dodecyl sulfate–acrylamide gels.

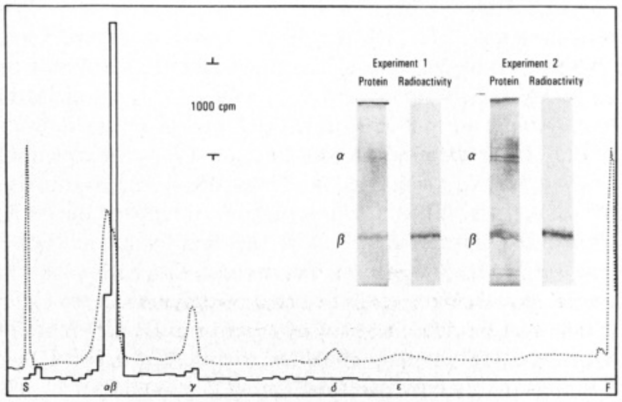

FIGURE 4: Distribution of [^{14}C]DCCD among the subunits of F_1. Conditions of incubation of F_1 with [^{14}C]DCCD, electrophoresis, and analysis of protein bands for radioactivity have been described under Materials and Methods. The amount of [^{14}C]DCCD-treated F_1 placed on the gel was 22 μg, and the amount of [^{14}C]DCCD bound to F_1 after two successive Sehadex column filtrations was 2.0 mol of DCCD/mol of F_1 (the molecular weight used was 350 000). The specific activity of the DCCD-treated F_1 was 0.5 μmol of ATP min^{-1} (mg of protein)$^{-1}$. (···) Densitometric trace of the acrylamide gel stained for protein with Coomassie blue; (—) radioactivity; S and F, the start and dye front of the acrylamide gel, respectively; α, β, γ, δ, and ε, the banding positions of the subunits of F_1-ATPase as shown on the dotted trace. Inset: Distribution of [^{14}C]DCCD radioactivity between the α and β subunits of F_1 analyzed by isoelectric focusing and autoradiography as described under Materials and Methods. In experiments 1 and 2, the amounts of [^{14}C]DCCD bound to F_1 after two successive Sephadex column filtrations were, respectively, 1.65 and 1.0 mol/mol of F_1.

to the V_{max} of the heat-activated enzyme, while the three K_m values remained essentially the same. The closed circles in Figure 1A,B show the effect of 10 mM $KHCO_3$ in the assay mixture. As seen in Table I, the stimulation by bicarbonate resulted in deletion of the high K_m. The data could be analyzed for two slopes with little or no change in $K_{m,1}$, $K_{m,2}$, and $V_{max,1}$, but with higher $V_{max,2}$. Previous analyses of kinetic data by double-reciprocal plots had indicated that bicarbonate abolishes the negative cooperativity with respect to ATP (Ebel & Lardy, 1975). It is, however, clear from the data of Figure 1 and Table I that this is true only in the regions of $K_{m,2}$ and $K_{m,3}$ but not at very low substrate concentrations. Similar results were obtained with ITP as substrate (Figure 2). The results clearly showed only two slopes with K_m values of 10^{-7} and 9×10^{-4} M with corresponding V_{max} values of 0.1 and 100 μmol min^{-1} (mg of protein)$^{-1}$.

Effect of Depletion of F_1 with respect to Tightly Bound Nucleotides. It has been suggested (Ohta et al., 1980; Tamura & Wang, 1983) and recently disputed (Meyers & Boyer, 1983) that the tightly bound nucleotides of F_1 on the α subunits have a regulatory function. Thus, it was of interest to see whether the tightly bound nucleotides of F_1 exert an influence on the negative cooperativity of the catalytic sites with respect to [ATP]. Two sets of experiments were done in this regard. In one experiment, bovine F_1 was rendered free of tightly bound nucleotides by the method of Garrett & Penefsky (1975). The resulting F_1 showed an A_{280}:A_{260} absorbance ratio of 1.86, suggesting that it was essentially nucleotide free [the theoretical A_{280}:A_{260} for nucleotide-free F_1 is 1.9; see Garrett & Penefsky (1975)]. The Eadie–Hofstee plot of the kinetics of ATP hydrolysis by this preparation could be fitted into two lines with the calculated K_m and V_{max} values shown in Table II. The second experiment involved the use of F_1-ATPase from the thermophilic bacterium PS3 (TF_1), which is considered to lack tightly bound nucleotides (Yoshida, 1983). However, in the case of TF_1, the best fit of the data was for three lines, for which the K_m and V_{max} values are also given in Table II together with the analysis of data for TF_1 assayed in the presence of 10 mM $KHCO_3$. The results of Table II indicate that the removal of tightly bound nucleotides from F_1, or their absence in TF_1, does not abolish the negative cooperativity with respect to [ATP] or the stimulatory effect of bicarbonate. They also show that the K_m values of TF_1 are in the same range as those of beef heart F_1, even though TF_1 is much less active than its mitochondrial counterpart.

Effect of Partial Inhibition of F_1 by Modification of β Subunits. As mentioned earlier, the catalytic sites of F_1 interact functionally in such a manner that results in positive catalytic cooperativity. Thus, it was of interest to see how the chemical modification of one or two catalytic subunits might affect the kinetics of the enzyme. For this purpose, DCCD was used as the modifier because of its covalent and stable interaction, target specificity (β subunit), and availability in radioactive form. As seen in Figure 3, the interaction of DCCD with F_1 also exhibits "negative cooperativity" in the sense that binding of approximately 1 mol of DCCD/mol of F_1 inhibited ATPase activity linearly up to about 90–95%, while the remainder of the activity was inhibited in small increments as DCCD binding increased up to and beyond 2 mol of DCCD/mol of F_1. Analysis of F_1 modified with [^{14}C]DCCD showed that up to about 1.5 mol of [^{14}C]DCCD/mol of F_1 the binding was exclusively on the β subunit, and at about 2–2.5 mol of [^{14}C]DCCD/mol of F_1, there was at

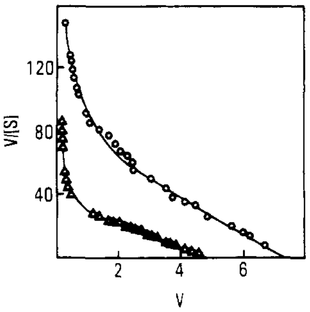

FIGURE 5: Eadie–Hofstee plots of the kinetics of ATP hydrolysis by [^{14}C]DCCD-modified F_1. Conditions for labeling of F_1 with [^{14}C]-DCCD have been described under Materials and Methods. The amounts of [^{14}C]DCCD bound to F_1 after two successive Sephadex column filtrations were 0.98 (circles) and 1.14 (triangles) mol/mol of F_1. The concentration range of ATP in the assay media was 2–4500 μM, and the concentration of F_1 was 7 μg/mL. With the DCCD-treated F_1 preparations used in these experiments, there was a linear relationship between the rate of ATP hydrolysis and enzyme concentration at least up to 8 μg of enzyme/mL of the assay medium.

best a marginal amount of radioactivity associated also with the γ subunit (Figure 4). The bound radioactivity was stable and as shown in Figure 3 was not altered by subjecting the modified enzyme to gel electrophoresis in the presence of sodium dodecyl sulfate. When a preparation of F_1 containing approximately 1 mol of DCCD/mol was assayed for activity as a function of [ATP], the results shown in Figure 5 were obtained. Both curves could be analyzed in terms of only two slopes, of which the K_m and V_{max} values for the preparation containing 1.14 mol of [^{14}C]DCCD/mol of F_1 are shown in Table I. The data indicate that $K_{m,1}$ and $K_{m,2}$ are essentially unchanged, $K_{m,3}$ is deleted, and the overall V_{max} is inhibited by about 95%. Thus, it is interesting to compare the results for the DCCD-modified F_1 with the data for unmodified F_1 assayed in the presence of bicarbonate. In both cases, the Eadie–Hofstee plots could be fitted by only two slopes (Table I, columns 6 and 3, respectively). In the DCCD-modified enzyme, exhibiting only 5% of the overall V_{max} activity, there are presumably only two functioning β subunits per F_1 molecule, which seems to agree with the fact that the kinetic data yielded only two K_m values. However, it appears that elimination of one functioning β subunit by chemical modification greatly diminishes overall turnover. In the unmodified F_1 assayed in the presence of bicarbonate, there are presumably three functioning active sites per F_1 molecule. Thus, we interpret the analyses for the latter experiments to mean that in the presence of bicarbonate the high K_m decreases and becomes essentially indistinguishable from the intermediate K_m. In addition, bicarbonate increases the overall V_{max} of F_1, an effect which is also seen with TF_1 without the elimination of $K_{m,3}$ (Table II).

Discussion

It has been shown that Eadie–Hofstee plots of the kinetics of ATP hydrolysis by F_1-ATPase can be analyzed best in terms of three K_m and V_{max} values when the rate studies are performed over a wide range of substrate concentrations. For the reasons discussed under Results, the calculated values for $K_{m,2}$ and $V_{max,2}$ are the most reliable, and $V_{max,3}$ is also in the right range, while $K_{m,1}$, $V_{max,1}$, and $K_{m,3}$ are the least precise because reliable experimental points could not be obtained at extremely low or high [ATP]. With this note of caution in mind, it might be concluded, therefore, that the overall results of Table I suggest that $K_{m,1}$ is in the micromolar range (i.e., <10 μM), $K_{m,2}$ is about 150–200 μM, and $K_{m,3}$ is in the millimolar range (i.e., <10 mM). $V_{max,2}$ and $V_{max,3}$ are of the order of several decamicromoles per minute per milligram, with $V_{max,2}$ being probably somewhat larger than $V_{max,3}$, while $V_{max,1}$ is of the order of micromoles per minute per milligram, possibly very much smaller. In general, the K_m and V_{max} values published by others for trisite catalysis by F_1 are in the same range as our $K_{m,2}$ and overall V_{max} (i.e., $V_{max,1+2+3}$) values, respectively. For $K_{m,1}$ and $V_{max,1}$, our results agree quite well with those of Gresser et al. (1982), who reported a K_m = 1.7 μM and a V_{max} = 2.2 μmol min^{-1} mg^{-1} as derived from a biphasic rate response curve.

However, it is important not to confuse $K_{m,1}$ and $V_{max,1}$ with the kinetic constants for unisite ATP hydrolysis. Grubmeyer et al. (1982) and Cross et al. (1982) have published a unisite association constant of 10^{12} M^{-1} and a V_{max} of 10^{-4} s^{-1}. This rate, which corresponds to 17 pmol min^{-1} mg of F_1^{-1}, was obtained for a single turnover with the ATP concentration deliberately chosen to be only one-third the concentration of F_1. Under these conditions, the off rate of ADP from F_1 was the controlling factor. Our rate measurements involved, however, multiple turnovers under conditions that substrate concentration (1–5000 μM) was always considerably greater than that of the enzyme (nanomolar). Thus, the two situations are not comparable, and the V_{max} of 10^{-4} s^{-1} is probably the correct rate estimate for a single turnover unisite ATP hydrolysis by F_1. Under our conditions, there could be effects on the catalytic properties of any one site by substrate binding to other catalytic or even possible regulatory sites. It is also possible that under multiple turnover conditions the enzyme exhibits a higher activity as compared to a single turnover situation. This possibility is analogous to the greater turnover rate of "pulsed" cytochrome *c* oxidase (reduced oxidase exposed to a pulse of oxygen) as compared to that of the resting enzyme (Antonini et al., 1977; Brunori et al., 1979; Kumar et al, 1984). Regardless of these considerations, however, it is clear that the kinetic data in the ATP concentration range employed could be analyzed best in terms of three different K_m and V_{max} values for both F_1 and TF_1 with roughly comparable magnitudes of the respective $K_{m,1}$, $K_{m,2}$, and $K_{m,3}$ values of the two enzymes. More significantly, it is seen in Table I that the various analyses and conditions had essentially no effect on the K_m values even when $K_{m,3}$ was deleted in certain instances.

It was shown originally by Ebel & Lardy (1975) that a number of anions (such as $HSeO_3^-$, HSO_3^-, chromate, bicarbonate, malonate, etc.) stimulate the activity of isolated rat liver F_1-ATPase. They also showed that increasing the concentrations of these ions in the assay medium gradually eliminated the downward trend of double-reciprocal ($1/v$ vs. 1/[MgATP]) plots as substrate concentration increased (negative cooperativity with respect to [MgATP]). For bicarbonate, addition of ≥10 mM $KHCO_3$ to the assay medium caused the highest rate enhancement and complete elimination of negative cooperativity insofar as could be detected by double-reciprocal plots. Similar results are obtained with beef heart ATPase. However, a more thorough analysis of the kinetics of ATP hydrolysis in the presence of 10 mM bicarbonate (Table I) indicated that the negative cooperativity with respect to [ATP] is eliminated only for the intermediate and high K_m values, but not entirely. Thus, in the presence of 10 mM bicarbonate, Eadie–Hofstee plots of the kinetics of ATP hydrolysis by F_1 (Figure 1, closed circles) could be analyzed in terms of only two slopes. The high $K_{m,3}$ was eliminated, $K_{m,1}$, $K_{m,2}$, and $V_{max,1}$ were essentially unchanged, and the overall V_{max} was increased by about 50% as compared to the kinetics of ATP hydrolysis in the absence of bicarbonate in the assay (Table I). In view of the results with the DCCD-modified F_1, the bicarbonate effect can perhaps be interpreted to mean that in the presence of this anion $K_{m,3}$ decreases and becomes indistinguishable from $K_{m,2}$, with an attendant increase in $V_{max,2} + V_{max,3}$. Essentially similar results were obtained with ITP as the substrate, which is also considered not to exhibit negative cooperativity (Ebel & Lardy, 1975). The kinetics of ITP hydrolysis yielded only two K_m values differing by about 10^4-fold.

Of particular interest is the result with F_1 which had been modified by DCCD. In this preparation, the enzyme contained 1 mol of DCCD/mol of F_1 bound exclusively to the β subunit, and the kinetic data clearly demonstrated a biphasic rate response curve with two K_m values. The latter were in the same range as the low and intermediate K_m values of the unmodified F_1, and the eliminated K_m was the one in the millimolar range. These results suggest, therefore, that the three K_m values determined for the unmodified enzyme are somehow related to the three catalytic sites, because elimination of one functional β subunit per F_1 molecule by DCCD modification reduced the number of K_m values to two with little or no change in their magnitude. The data also show the effect of DCCD modification of one β subunit per F_1 molecule on the activity of the remaining functional sites. Thus, as compared to its unmodified control, the activity of the two-site enzyme was diminished by 95%, which agrees with the phenomenon of catalytic cooperativity demonstrated by Cross et al. (1982) and Gresser et al. (1982).

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