Substrate Metal–Adenosine 5′-Triphosphate Chelate Structure and Stereochemical Course of Reaction Catalyzed by the Adenosinetriphosphatase from the Thermophilic Bacterium PS3†

Peter Senter,‡ Fritz Eckstein,* and Yasuo Kagawa

ABSTRACT: The structure of the active metal–nucleotide complex in TF_1 ATPase has been determined by using phosphorothioate analogues of ATP with Mg^{2+} and Cd^{2+} as the activating metal ions. Both diastereomers of adenosine 5′-*O*-(1-thiotriphosphate) (ATPαS) were substrates for TF_1, and no metal-dependent diastereomeric selectivity was observed. Mg·(S_P)-ATPβS and Cd·(R_P)-ATPβS, which have the Δ-chelate structure, were better substrates than Mg·(R_P)-ATPβS and Cd·(S_P)-ATPβS, both of which have the Λ-chelate structure. These results suggest that TF_1 uses the Δ,β,γ-bidentate nucleotide chelate as substrate. (R_P)-[βγ-^{18}O,γ-^{18}O]ATPγS was hydrolyzed by TF_1 in oxygen-17-labeled water, and the product inorganic [^{16}O,^{17}O,^{18}O]thiophosphate was shown to have the R_P configuration. The reaction thus proceeds with inversion of configuration at phosphorus, and a direct in-line reaction mechanism is indicated.

The proton-translocating ATPase[1] F_1F_0 catalyzes the formation of ATP from ADP and phosphate by utilizing the electrochemical gradient of protons derived from the electron-transfer chain. The F_1 portion of the complex has ATPase activity and is bound to the F_0 portion, which functions as a proton channel. The F_1F_0 complex has the same basic structure in various energy-transducing membranes such as mitochondria, chloroplasts, and bacteria. The F_1 portion has been isolated from the thermophilic bacterium PS3, which is indigenous to a hot spring in the Shizuoka prefecture of Japan. In the soluble form, the main enzyme activity is ATP hydrolysis. The advantages of using thermophilic F_1 (TF_1) for mechanistic studies is its extreme stability (Yoshida et al., 1975). Thus, TF_1 can be reconstituted from its subunits after complete destruction of their secondary structure (Yoshida et al., 1979), the effects of nucleotide binding to the isolated α- and β-subunits can be measured (Ohta et al., 1980), and its ATP-synthesizing activity can be assayed under a variety of conditions (Kagawa, 1982). Compared with other F_1s, divalent metal specificity of TF_1 is low (Yoshida et al., 1975). It is this latter property that suggested to us to try to identify the structure of the Mg·ATP substrate by the phosphorothioate method (Cohn, 1982; Eckstein, 1979, 1983; Frey, 1982a,b) since it ideally requires good enzymatic activity in the presence of Mg^{2+} as well as Cd^{2+}.

Mechanistically, little is known about the enzyme. An understanding of the mechanism of TF_1-catalyzed ATP hydrolysis requires knowledge of both the stereochemical course of the reaction and the structure of the metal–ATP chelate. Stereochemical investigations have been undertaken for the reactions catalyzed by myosin (Webb & Trentham, 1980) and mitochondrial ATPase (Webb et al., 1980), both of which proceed with net inversion of configuration at the γ-phosphate of the ATP substrate. In contrast, sarcoplasmic reticulum ATPase catalyzes ATP hydrolysis with retention of configuration, providing evidence for an enzyme–phosphate intermediate (Webb & Trentham, 1981). The active metal–chelate structure for ATP in the active site of myosin ATPase has been investigated with the diastereomers of ATPαS and ATPβS (Connolly & Eckstein, 1982).

Such mechanistic investigations for TF_1 ATPase would not only detail the nature of the enzyme active site but also serve to correlate this enzyme with the other known ATPases. This paper describes the use of chiral ATP phosphorothioates for the determination of the chelate structure of the active metal–ATP complex and for the determination of the stereochemical course of the hydrolysis reaction.

Materials and Methods

Materials. TF_1 was purified to homogeneity from the thermophilic bacterium PS3 as previously described (Kagawa & Yoshida, 1979). The enzyme was dialyzed against deionized water before lyophilization. About 3 h before all kinetic experiments, the enzyme was dissolved in 50 mM glycine–NaOH buffer at pH 9.4, dialyzed against the same buffer containing 1 mM EDTA, and then dialyzed against the buffer containing suspended Chelex 100-Na^+ resin. The protein concentration was determined according to the method of Mejbaum-Katzenellenbogen & Dobryszycka (1959) with bovine serum albumin as a reference and measurement in quartz cuvettes at 550 nm.

The following enzymes were obtained from Boehringer Mannheim: glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle, 100 units/mg), phosphoglycerate kinase (yeast, 450 units/mg), aldolase (rabbit muscle, 9 units/mg), triosephosphate isomerase (yeast 10000 units/mg), lactate dehydrogenase (pig muscle, 550 units/mg), myokinase (rabbit muscle, 360 units/mg), and hexokinase (yeast, 140 units/mg). All enzymes obtained as ammonium sulfate suspensions were dialyzed against 20 mM Tris-HCl buffer, pH 8.0, containing 1 mM dithiothreitol and 1 mM EDTA for 2 h before use.

† From the Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, D-3400 Göttingen, West Germany (P.S. and F.E.), and the Department of Biochemistry, Jichi Medical School, Minamikawachi-Machi, Tochigi-Ken, Japan 329-04 (Y.K.). *Received May 31, 1983.*

‡ Recipient of a research fellowship from the Alexander von Humboldt Foundation. Present address: Department of Tumor Immunology, Dana-Farber Cancer Institute, Boston, MA 02115.

[1] Abbreviations: ATPase, adenosinetriphosphatase; AMPS, adenosine 5′-phosphorothioate; (R_P)- and (S_P)-ATPαS, R_P and S_P diastereomers of adenosine 5′-*O*-(1-thiotriphosphate); (R_P)- and (S_P)-ATPβS, R_P and S_P diastereomers of adenosine 5′-*O*-(2-thiotriphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); HPLC, high-pressure liquid chromatography; TF_1, thermophilic F_1 (soluble ATPase from thermophilic bacterium PS3); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance.

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[^{35}S]ATPγS (250 Ci/mmol) and Aquasol were purchased from New England Nuclear, and [γ-^{32}P]ATP and [^{32}P]P_i were purchased from Amersham Corp.

The following compounds were prepared according to previously described procedures, and their purities were confirmed by HPLC and ^{31}P NMR: (S_P)-ATPαS (Jaffe & Cohn, 1978), (R_P)-ATPαS and (S_P)-ATPβS (Yee et al., 1979), (R_P)-ATPβS (Eckstein & Goody, 1976), (S_P)-[γ-^{32}P]ATPβS (Romaniuk & Eckstein, 1981), (R_P)-[γ-^{32}P]ATPβS (Stingelin et al., 1980), [$^{18}O_2$]AMPS and (R_P)-[βγ-^{18}O,γ-^{18}O]ATPγS (Richard & Frey, 1982), and (S_P)-[α-^{18}O,αβ-^{18}O]ADPαS (Webb & Trentham, 1980). 2′,3′-(Methoxymethylidene)AMP was prepared according to the method of Webb & Trentham (1980) but 1.3 equiv of *p*-toluenesulfonic acid was used instead of HCl as the acid catalyst. After 45 min at room temperature, the clear solution was neutralized with triethylamine and evaporated, and the product was purified by DEAE-Sephadex chromatography. The yields ranged from 66 to 87%. $H_2{}^{17}O$ (containing 9.4% ^{16}O, 52.8% ^{17}O, and 37.8% ^{18}O) was obtained from Monsanto Research Corp. (Miamisburg, OH), and $H_2{}^{18}O$ (98.4% $H_2{}^{18}O$) was obtained from Ventron (Karlsruhe, West Germany).

^{31}P NMR spectra were recorded on a Bruker WP 200 SY spectrophotometer operating at 81.01 MHz with quadrature detection and broad-band proton decoupling with 85% aqueous H_3PO_4 as external standard. Chemical shifts are given in ppm and are positive when downfield from this standard. HPLC experiments were performed with a Waters Associates (Model 6000 A) liquid chromatograph using the following columns and solvent systems: anion exchange (Nucleosil 10SB, Macherey-Nagel, Düren, West Germany) eluting with 200 mM KH_2PO_4 containing 300 mM KOAc at pH 4.5; reverse phase (ODS Hypersil, 5 μm, Shandon Southern Products Ltd., Runcorn, England) eluting with 500 mM KH_2PO_4 at pH 6.0.

General Procedures for Determination of Kinetic Constants for TF_1. In the following assay procedures, all substrates and solutions were passed at least twice through a column containing Chelex 100-Na^+ resin. Stock solutions of $Cd(OAc)_2$ and $MgCl_2$ were prepared from water that was deionized with Chelex 100-Na^+ resin. Control experiments included assays without enzyme and without added metal, and in these experiments no hydrolysis of ATP was observed. All assays were repeated 2–5 times, and the kinetic constants were derived from Lineweaver–Burk plots analyzed by least-squares analyses.

Assay of TF_1 Activity by HPLC. The assay solutions at 37 °C contained in volumes ranging from 150 to 500 μL 0.17–0.80 mM nucleotide, 150 mM glycine–NaOH buffer at pH 9.4, and 1 mM $MgCl_2$ or 5 mM $Cd(OAc)_2$. The reactions were initiated by the addition of TF_1 dissolved in 50 mM glycine–NaOH at pH 9.4. Enzyme concentrations ranged from 3 μg/mL for Mg·ATP to 485 μg/mL for Cd·(R_P)-ATPαS. Aliquots were quenched within the linear range of product formation by addition of an equal volume of ice-cold 2 N NaOH. The percentage of product formation was determined from the relative peak areas from anion-exchange HPLC.

Assay of TF_1 Activity with ^{32}P- and ^{35}S-Labeled Nucleotides. The assay solutions at 37 °C contained in a total volume of 250 μL 0.12–0.84 mM nucleotide, 150 mM glycine–NaOH buffer at pH 9.4, and 1 mM $MgCl_2$ or 5 mM $Cd(OAc)_2$. The enzyme was added as before, so that the final concentration ranged from 5.4 μg/mL for Mg·ATP to 920 μg/mL for Mg·(R_P)-ATPβS. Aliquots (50 μL) were withdrawn at three different time points and added to an ice-cold suspension of 100 mg of activated charcoal in 2 mL of 50 mM KH_2PO_4 (50 mM Na_3PSO_3 for [^{35}S]ATPγS) and 20 mM EDTA. After centrifugation (10 min, 6000 rpm at 4 °C), 0.5 mL of the supernatant was withdrawn and counted in Aquasol.

TF_1-Catalyzed Hydrolysis of (R_P)-[βγ-^{18}O,γ-^{18}O]ATPγS. A solution of 50 nmol of ATPγS containing 1 μCi of [^{35}S]-ATPγS and 50 μmol of (R_P)-[βγ-^{18}O,γ-$_{18}$O]ATPγS (Richard & Frey, 1982) was filtered through a column containing Dowex Na^+ resin and then lyophilized overnight. Another solution of 0.15 mL of 100 mM dithiothreitol, 50 μL of 100 mM $MgCl_2$, and 0.15 mL of 1 M glycine–NaOH at pH 9.4 was lyophilized separately. To each lyophilate was added 100 μL of $H_2{}^{17}O$, and the resulting solutions were relyophilized. The lyophilized buffer salts were dissolved in 0.5 mL of $H_2{}^{17}O$ and added to the ATPγS, and the reaction was initiated by the addition of 2 mg of solid TF_1. After 1.5 h at 37 °C, the reaction was complete as determined by anion-exchange HPLC. In a control experiment without any enzyme, no hydrolysis of ATPγS was observed. The solution was cooled to 3 °C, and 0.1 mL of 0.4 M EDTA was added to inhibit any further enzyme activity. Purification of the product on a 1 × 15 cm DEAE-Sephadex column with a linear gradient of 250 mL each of 10–300 mM triethylammonium bicarbonate afforded 31 μmol of thiophosphate, which was detected and quantified by the presence of the ^{35}S label. An aliquot of 4 μmol was methylated, and the ^{31}P NMR spectrum of this S-methylated derivative (Webb, 1982) showed an $^{18}O_2$ to ^{18}O ratio of 3.39, indicating that the isotopic composition of the thiophosphate was 11.0% ^{16}O, 51.9% ^{17}O, and 37.1% ^{18}O.

Incorporation of [^{16}O,^{17}O,^{18}O]Thiophosphate into (S_P)-ATPβS. The procedure used was similar to that reported by Webb (1982). All enzymes were dialyzed as previously described. The thiophosphate from enzymatic digestion (27 μmol) was converted to ATPγS at room temperature in a solution (30 mL) containing 5.31 mM ADP, 0.71 mM NAD^+, 2 mM $MgCl_2$, 2 mM dithiothreitol, 4.4 mM fructose 1,6-diphosphate, 10 mM sodium pyruvate, 100 mM Tris-HCl at pH 8.0, 1600 units of glyceraldehyde-3-phosphate dehydrogenase, 10 000 units of phosphoglycerate kinase, 28 units of aldolase, 2000 units of triosephosphate isomerase, and 400 units of lactate dehydrogenase. After 90 min, anion-exchange HPLC indicated quantitative formation of ATPγS. To this solution were added 125 mg of Na_2AMP (0.32 mmol) and 3600 units of myokinase. After 2.5 h, the solution was cooled to 0 °C, and 1 mL of 0.4 M EDTA was added to inhibit further enzyme activity. Purification of the product on a 3 × 30 cm DEAE-Sephadex column preequilibrated with 0.1 M triethylammonium bicarbonate and elution with a linear gradient of 1.5 L each of 0.1 and 0.5 M triethylammonium bicarbonate provided 27 μmol (100%) of [^{16}O,^{17}O,^{18}O]ADPβS, which was pure by HPLC analysis.

The [^{16}O,^{17}O,^{18}O]ADPβS was phosphorylated in a solution (25 mL) containing 10 mM KH_2PO_4, 5.45 mM fructose 1,6-diphosphate, 0.71 mM NAD^+, 21.8 mM sodium pyruvate, 2 mM $MgCl_2$, 2 mM dithiothreitol, 100 mM Tris-HCl at pH 8.0, 500 units of glyceraldehyde-3-phosphate dehydrogenase, 2000 units of phosphoglycerate kinase, 500 units of lactate dehyrogenase, 40 000 units of triosephosphate isomerase, and 28 units of aldolase. After 2 h at room temperature, the product was applied to a 1.5 × 30 cm DEAE-Sephadex column preequilibrated with 20 mM triethylammonium bicarbonate and eluted with a linear gradient of 0.5 L each of 0.2 and 0.6 M triethylammonium bicarbonate. A total of 23.3 μmol (86%) of [^{16}O,^{17}O,^{18}O]ATPβS was isolated and dissolved in 0.4 mL of 100 mM Tris-HCl at pH 8.0, 50 mM EDTA, and 10 mM

Table I: Kinetic Constants for ATP and the Phosphorothioate Analogues in the Presence of Mg^{2+} and Cd^{2+}

substrate	K_m (mM)	V_{max} [nmol/(min·mg)]	$V_{max}(S_P)/V_{max}(R_P)$
Mg·ATP[a,b]	0.84	4915	
Cd·ATP[b]	1.25	482	
Mg·(S_P)-ATPαS[a]	1.36	10550	753
Mg·(R_P)-ATPαS[a]	0.71	14	
Cd·(S_P)-ATPαS[a]	1.28	200	22
Cd·(R_P)-ATPαS[a]	0.95	9.0	
Mg·(S_P)-ATPβS[a,b]	1.81	11.5	≥575
Mg·(R_P)-ATPβS[a,b]		≤0.02	
Cd·(S_P)-ATPβS[b]	1.09	5.0	0.5
Cd·(R_P)-ATPβS[b]	1.30	9.9	
Mg·ATPγS[c]	0.61	1440	

[a] Calculated by using HPLC. [b] Calculated by using γ-^{32}P-labeled substrate. [c] Calculated by using γ-^{35}S-labeled substrate.

dithiothreitol and D_2O–H_2O, 3:1, for ^{31}P NMR measurement.

Results

TF_1 Activity with Diastereomers of ATPαS and ATPβS. Purified TF_1 has been shown to display ATPase activity with a wide range of divalent metal ions (Yoshida et al., 1975). In this investigation, the concentrations of Mg^{2+} and Cd^{2+} metal ions of optimal activity were determined to be 1 and 5 mM, respectively. Traces of activity in the absence of added divalent metals were eliminated by passage of buffers and water through Chelex resin columns and by dialysis of the enzyme successively against EDTA and suspended Chelex resin.

A combination of analytical methods was used to obtain the kinetic constants for the TF_1-catalyzed hydrolysis of ATP and the phosphorothioate analogues. The values were obtained either by HPLC or by the measurement of radioactivity released from isotopically labeled substrates. Good agreement was obtained in cases where both methods were employed.

Table I shows that in the presence of Mg^{2+} and Cd^{2+}, both diastereomers of ATPαS were substrates for the enzyme. A higher level of activity was observed for (S_P)-ATPαS than for (R_P)-ATPαS. There was no metal-dependent reversal of diastereomeric specificity as determined by the S_P/R_P V_{max} ratios.

For the ATPβS diastereomers, a metal-dependent reversal of activity was observed. While Mg·(S_P)-ATPβS was a substrate for the enzyme, the Mg·(R_P)-diastereomer was not a substrate at all. In contrast, Cd·(R_P)-ATPβS proved to be a better substrate than the S_P diastereomer. The observed metal-dependent reversal of activity for the ATPβS diastereomers provides evidence that the metal is complexed to the β-phosphate of ATP in the enzyme active site during the rate-limiting step of hydrolysis.

Determination of Stereochemical Course of Reaction. The TF_1-catalyzed hydrolysis of Mg^{2+}·ATPγS proceeds about one-third as fast as Mg^{2+}·ATP hydrolysis (Table I). In order to determine the stereochemical course of reaction, it was first necessary to demonstrate the absence of oxygen scrambling between the thiophosphate product and water. This was achieved by enzymatic hydrolysis of ATPγS in oxygen-18-labeled water. The thiophosphate product was analyzed as the *S*-methyl ester by ^{31}P NMR spectroscopy according to the procedure of Webb (1982) and was shown to contain only one oxygen-18 atom. Therefore, no exchange of oxygen had taken place.

The stereochemical course of the TF_1-catalyzed hydrolysis reaction was determined by using (R_P)-[βγ-^{18}O,γ-^{18}O]ATPγS as substrate (Richard & Frey, 1982). The hydrolysis was

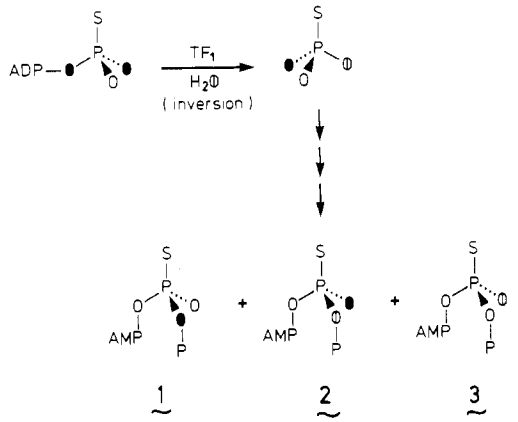

FIGURE 1: Stereochemistry and stereochemical analysis of [^{16}O,^{17}O,^{18}O]thiophosphate produced by TF_1 ATPase. (R_P)-[βγ-^{18}O,γ-^{18}O]ATPγS (^{18}O, ●) was hydrolyzed in $H_2{}^{17}O$ (^{17}O, Φ). ADP, adenosyl 5′-diphosphoryl; AMP, adenosine 5′-phosphoryl.

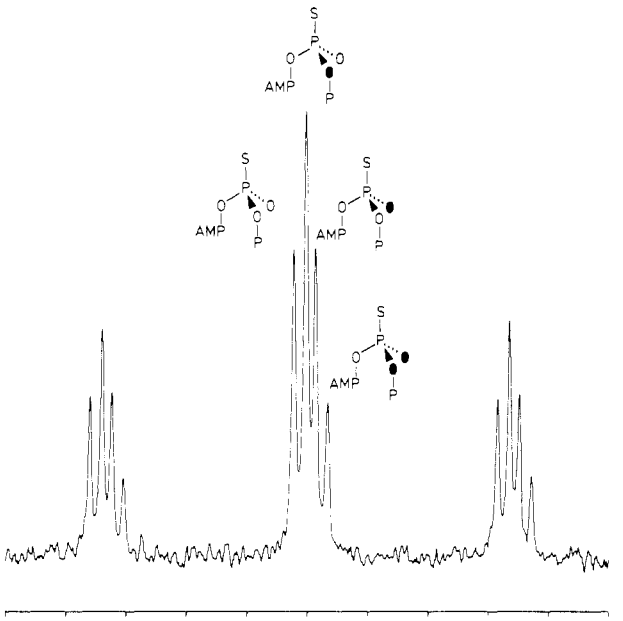

FIGURE 2: ^{31}P NMR spectrum of isotopically labeled (S_P)-ATPβS. The spectrum was obtained at 81.01 MHz. Spectra width 800 Hz; acquisition time 10.24 s; pulse width 21 μs; number of transients 1158. The chemical shifts of the four resonances of the central quadruplet are 29.5240, 29.5037, 29.4889, and 29.4682 ppm. The scale is 0.1 ppm per division, (^{18}O, ●).

conducted in oxygen-17-enriched water, yielding [^{16}O,^{17}O,^{18}O]thiophosphate as product. NMR analysis of the *S*-methyl ester of the product showed that there was no excess oxygen-17 from oxygen-exchange reactions.

The absolute configuration of the inorganic [^{16}O,^{17}O,^{18}O]thiophosphate was assigned by the method of Webb & Trentham [1980; see also Webb (1982)]. This method involves the incorporation of the isotopically labeled thiophosphate into (S_P)-ATPβS with enzymes having known stereochemical reaction courses (Figure 1). A mixture of isotopically labeled (S_P)-ATPβS isomers was obtained in a yield of 53% from oxygen-18-labeled ATPγS. The ^{31}P NMR spectrum of P_β revealed the triplet of quartets shown in Figure 2. As will be discussed, the major peak arises from species **1**, and the other peaks are due to H_2O and $H_2{}^{18}O$ present in the oxygen-17-labeled water and to isotope washout in the synthesis of ATPβS from thiophosphate. This indicates that the thiophosphate obtained from TF_1-catalyzed hydrolysis of (R_P)-[βγ-^{18}O,γ-^{18}O]ATPβS has the R_P configuration and that

the hydrolysis proceeded with inversion of configuration.

Discussion

The objectives of this investigation have been to establish the structure of the metal–nucleotide chelate in the active site of TF_1 ATPase and to determine the stereochemical course of the reaction. This gives both detailed information concerning the mechanism of reaction and also provides a basis upon which the various ATPases may be compared. Related investigations have provided a wealth of information for enzyme-catalyzed reactions at phosphorus [for review, see Eckstein (1979, 1983), Knowles (1980), Frey (1982a,b), Cohn (1982), and Eckstein et al. (1982)].

The diastereomers of ATPαS and ATPβS were prepared in both radioactively labeled and unlabeled forms according to previously published procedures. The absolute configurations of these compounds are known (Eckstein, 1979, 1983). The chelation of various metals to the ATP phosphorothioate analogues has been investigated (Cohn, 1982; Jaffe & Cohn, 1978), and it has been shown that Mg^{2+} preferentially coordinates to oxygen while Cd^{2+} coordinates to sulfur. As a result, Mg·(S_P)-ATPβS and Cd·(R_P)-ATPβS have the same metal β,γ-bidentate chelate structure [designated Δ according to Cornelius & Cleland (1978)], and the opposite chelate structure (designated Λ) is found for Mg·(R_P)-ATPβS and Cd·(S_P)-ATPβS. A corresponding situation exists for ATPαS where Mg·(S_P)-ATPαS and Mg·(S_P)-ATPαS and Cd·(R_P)-ATPαS have the same α,β-bidentate chelate structure, designated Λ in this case.

A reversal of metal-dependent diastereomeric selectivity as measured by the V_{max} S_P/R_P ratios is an indication that the metal is coordinated to the phosphate in question. Such reversals of stereoselectivity for the phosphorothioates of ATP have been applied to the determination of the metal–ATP complex of a variety of enzyme reactions (Eckstein, 1979, 1983; Cohn, 1982; Frey, 1982a,b).

Both diastereomers of ATPαS were substrates for TF_1, and the V_{max} S_P/R_P ratios did not show a metal-dependent reversal although the ratio decreased from 753 in the presence of Mg^{2+} to 22 in the presence of Cd^{2+}. This difference of a factor of 34 is unusually large for a phosphorothioate group where no reversal is being observed. The most likely interpretation of this results is that the metal is not coordinated to the α-phosphate in the enzyme active site. However, since other factors such as geometric constraints within the enzyme active site may lead to lack of reversal (Cohn, 1982; Eckstein, 1983), investigations with Mg·(S_P)-ATPαSCH_3 were undertaken. In this analogue, the α-phosphate group exists as a phosphate triester, and so its ability to chelate with metals is severely reduced (Connolly & Eckstein, 1982). If ATPαSCH_3 were a substrate for the enzyme, this would support the interpretation that metal coordination to α-phosphate is not involved in the enzymatic reaction. Unfortunately, experiments with this analogue were complicated by the fact that the enzyme-catalyzed hydrolysis was only marginally faster than the nonenzymatic reaction so that these results could not be unambiguously interpreted.

In contrast, the diastereomers of ATPβS showed a metal-dependent reversal of activity. Mg·(S_P)-ATPβS and Cd·(R_P)-ATPβS, both having the Δ-chelate structure, proved to be better substrates than their respective Λ-chelate analogues (Table I). However, the V_{max} S_P/R_P ratio for the Cd·ATPβS proved to be higher than expected, indicating that Cd·(S_P)-ATPβS still is a substrate. This may be due to constraints in the enzyme active site, which force the normally unfavorable Cd → O coordination. Similar incomplete reveral of stereoselectivity has also been observed for other enzymes (Cohn, 1982; Eckstein, 1983). The observed reversal for the ATPβS diastereomers indicates that the metal is complexed to the β-phosphorothioate at some stage of the enzymatic reaction, presumably during the rate-limiting step. Since no reversal is found at α-phosphate, the interpretation of this results is that the metal in addition to the β-phosphate has to be complexed to the γ-phosphate. This complexation to the γ-phosphate cannot be shown by the phosphorothioate method directly, but this β,γ-bidentate structure is assumed on the basis of its higher themodymanic stability. The conclusion then is that TF_1 ATPase requires the β,γ,Δ-metal–ATP chelate structure. This same structure of the metal–ATP complex has also been found for the substrate in the myosin ATPase reaction (Connolly & Eckstein, 1981).

Of other ATPases associated with energy-transducing membranes, the spinach chloroplast as well as the beef heart mitochondrial ATPases have been studied with respect to the structure of the metal–ATP complex. Thus, Strotmann et al. (1979) have shown that Mg·(S_P)-ATPαS and Mg·(S_P)-ATPβS were better substrates for the light-triggered ATPase of spinach chloroplasts than the corresponding R_P diastereomers. Unfortunately, these data cannot be interpreted in terms of a metal–ATP chelate structure because they were carried out in the presence of one metal ion only, but they are in agreement with those reported here for the TF_1 ATPase. On the basis of studies with exchange-inert Cr·ADP and Cr·ATP complexes (Cleland, 1982), Frasch & Selman (1982) have suggested an α,β,γ-tridentate metal–ATP complex as substrate for the spinach chloroplast F_1 ATPase. For the beef heart mitochondrial ATPase, it was found that only the mono- and tridentate Cr·ATP complexes and the monodentate Cr·ADP complex were competitive inhibitors for the ATPase reaction (Bossard & Schuster, 1981). These results suggested to the authors that monodentate Mg·ATP might be the substrate for ATP hydrolysis and bidentate Mg·ATP the ATP synthesis product.

The stereochemical course of the reaction with (R_P)-[$\beta\gamma$-^{18}O,γ-^{18}O]ATPγS as a substrate was determined by the procedure of Webb & Trentham (1980). The inorganic thiophosphate from enzymatic hydrolysis was enzymatically converted to (S_P)-ATPβS as shown in Figure 1. Because of the nuclear quadrupolar moment of oxygen-17, species **2** and **3** are not observed in the ^{31}P NMR spectrum (Tsai, 1979, 1982; Lowe et al., 1979). The presence of oxygen-18 causes an upfield shift in the ^{31}P resonance, and the magnitude of the shift is dependent upon the bond order (Lowe et al., 1979; Cohn & Hu, 1980; Lowe & Sproat, 1978). Therefore, species **1** which has a bridging P_β–O–P_γ oxygen-18 is easily distinguished from the corresponding terminal P_β oxygen-18 isomer since the upfield NMR perturbation of the former will be smaller.

The ^{31}P NMR spectrum of the β-phosphorus in ATPβS shows four distinct resonances (Figure 2). This is due to the isotopic composition of oxygen-17-labeled water (containing only 52.8% oxygen-17) and to the isotope washout in the conversion of thiophosphate to ATPβS (Webb, 1982). The major resonance, due to species **1**, indicates that the thiophosphate from enzymatic digestion had premoninantly the R_P configuration. On the basis of the composition of the oxygen-17-labeled water and the extent of isotope washout (approximately 50%), it is estimated that TF_1 catalyzes ATPγS hydrolysis with at least 80% inversion of configuration at phosphorus. A correspondingly high level of washout has been previously observed (Hansen & Knowles, 1982).

This result is most simply explained by a single nucleophilic substitution reaction at phosphorus in an in-line fashion (Eckstein, 1979, 1983; Frey, 1982a,b; Knowles, 1980; Buchwald et al., 1982) and argues against the existence of a covalent enzyme intermediate. If this existed, retention of configuration as the result of two displacement reactions would have been observed. However, as has been realized more recently, this simple interpretation can be more complicated if a carboxyl group participates in the catalysis of the reaction. This has been discussed in more detail for the reaction catalyzed by staphylococcal nuclease (Mehdi & Gerlt, 1982). The complication arises because an acyl–phosphate–enzyme intermediate can in principle be hydrolyzed by nucleophilic attack of H_2O on either phosphorus or carbon, the former resulting in retention and the latter in inversion of configuration.

Inversion of configuration has also been found for myosin (Webb & Trentham, 1980) and mitochondrial ATPase (Webb et al., 1980), but the sarcoplasmic reticulum ATPase catalyzed reaction proceeds with retention of configuration (Webb & Trentham, 1981). For this ATPase, a covalent acyl–phosphate–enzyme intermediate has been shown to exist (de Meis & Vianna, 1979), and thus hydrolysis of this intermediate must have occurred by nucleophilic attack of H_2O on phosphorus. If one takes this result as the basis for the interpretation of the results with the other ATPases, inversion would be, indeed, the result of a single direct nucleophilic substitution reaction.

Acknowledgments

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