Evidence That Catalysis by Yeast Inorganic Pyrophosphatase Proceeds by Direct Phosphoryl Transfer to Water and Not via a Phosphoryl Enzyme Intermediate†

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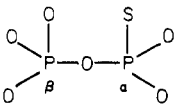
ABSTRACT: In this work, we show that adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) is a substrate for yeast inorganic pyrophosphatase (PPase) (EC 3.6.1.1) and further, using chirally labeled [γ-^{17}O,^{18}O]ATPγS, that enzyme-catalyzed hydrolysis to produce chiral inorganic thio[^{17}O,^{18}O]phosphate proceeds with inversion of configuration. Both the synthesis of chiral ATPγS and the determination of inorganic thiophosphate configuration were carried out as described by Webb [Webb, M. R. (1982) *Methods Enzymol. 87*, 301–316]. We also show in a single turnover experiment performed in $H_2{}^{18}O$ that 1 mol each of $^{18}O^{16}O_3P$ and $^{16}O_4P$ is produced per mol of inorganic pyrophosphate hydrolyzed, a strong indication that oxygen uptake to form inorganic phosphate on PPase catalysis of inorganic pyrosphosphate hydrolysis comes directly from H_2O. These two results provide strong evidence for the conclusion that PPase catalyzes inorganic pyrophosphate hydrolysis via a single-step direct phosphoryl transfer to water and does not involve formation of a phosphorylated enzyme intermediate.

The stereochemistry of enzyme-catalyzed phosphoryl transfer has been used in recent years to obtain evidence for or against an intermediate covalent phosphoryl enzyme (Knowles, 1980). Because all enzyme-catalyzed displacements at phosphoryl centers are believed to proceed with inversion, the result of overall inversion of configuration in an enzymatic reaction has been taken as evidence that the reaction proceeds via a single-step, direct transfer mechanism. By contrast, overall retention of configuration has been taken as evidence for two-step transfer via a covalent phosphoryl enzyme or other phosphorylated intermediate.

We recently proposed a kinetic mechanism for yeast inorganic pyrophosphatase (EC 3.6.1.1) (PPase)[1] catalysis of inorganic pyrophosphate (PP_i) hydrolysis which assumed single-step, direct transfer of a phosphoryl group to water (Springs et al., 1981; Welsh et al., 1983b), in line with the failure of several groups to find a phosphoryl enzyme intermediate (Rapoport et al., 1973; Sperow et al., 1973). However, none of this work provided definitive evidence against the formation of such an intermediate in small amounts during turnover. Moreover, Baykov et al. (1977) and Avaeva et al. (1977) presented strong evidence, on the basis of NaB^3H_4 quenching experiments, for the formation of a covalently phosphorylated aspartyl residue when enzyme is incubated with PP_i and fluoride ion. Although no evidence was presented for the kinetic competency of this intermediate in overall turnover, and in fact evidence has recently been presented that such phosphorylation does not take place at the active site (Bakuleva et al., 1981), the situation is sufficiently unclear that determination of the stereochemistry of PPase catalysis would be a valuable contribution. Thiophosphoryl phosphate (**1**), though a substrate, is unsuitable for such a study because its hydrolysis by PPase proceeds exclusively via H_2O attack on the β-



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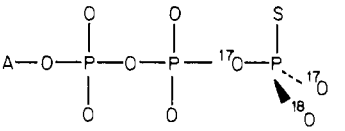
phosphoryl group (Webb & Trentham, 1980). However, we can exploit the known ATPase activity of PPase (Schlesinger & Coon, 1960) to obtain hydrolysis of [β,γ-^{17}O,γ-^{17}O,^{18}O]-ATPγS (**2**) to ADP and inorganic thiophosphate (SP_i) via



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attack on the chiral γ-thiophosphoryl group. In this report, we determine the configuration of the SP_i produced on PPase-catalyzed chiral ATPγS hydrolysis and find that hydrolysis proceeds with inversion. We also demonstrate, in a single turnover experiment performed in $H_2{}^{18}O$, that 1 mol each of $^{18}O^{16}O_3P$ and $^{18}O_4P$ is produced per mol of PP_i hydrolyzed. These two results provide strong evidence that PPase catalysis proceeds via direct, single-step transfer of a phosphoryl group from PP_i to water.

Experimental Procedures

Materials

PPase was prepared and its subunit concentration measured as described previously by Welsh et al. (1983a). The following materials were obtained from the sources indicated: carrier-free [^{32}P]PP_i, New England Nuclear; ^{18}O-enriched water (98%), Prochem; ^{17}O-enriched water (51%), Monsanto; Dowex 1-X2 100 mesh, Bio-Rad. [β,γ-^{17}O,γ-^{17}O,^{18}O]ATPγS (S_p

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[1] Abbreviations: DTT, dithiothreitol; HPLC, high-performance liquid chromatography; Mes, 2-(*N*-morpholino)ethanesulfonate; P_i, inorganic phosphate; PPase, yeast inorganic pyrophosphatase; PP_i, inorganic pyrophosphate; SP_i, inorganic thiophosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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isomer) was synthesized as described by Webb (1982).

Methods

ATPγS and SP_i Hydrolysis. Rates of ATPγS hydrolysis were followed by measuring ATPγS disappearance by HPLC (Eccleston & Webb, 1982). Aliquots containing 1 nmol of nucleotide were quenched prior to injection with 0.1 mL of HPLC eluting buffer (0.7 M ammonium phosphate, pH 4.0). Rates of PPase catalysis of SP_i hydrolysis were followed by measuring P_i formation using a modified Fiske–Subbarow method (Josse, 1966).

Stereochemistry of ATPγS Hydrolysis. [β,γ-^{17}O,γ-^{17}O,^{18}O]ATPγS (S_p isomer) (20 μmol) was hydrolyzed at 37 °C in 10 mL of 100 mM Mes buffer (pH 6.7) which also contained 0.5 mM $ZnCl_2$, 0.5 mM $MnCl_2$, 2 mM DTT, and PPase (specific activity 250 μmol of PP_i hydrolyzed mg^{-1} min^{-1}; 2 mg/mL). The incubation was quenched with 2 g of Dowex 50 (H^+ form) after 6 h, and the SP_i formed was isolated by ion-exchange chromatography at 4 °C on a DEAE-cellulose column as described (Webb, 1982). The configuration of the product SP_i was determined by incorporating it into ATPβS, which was then analyzed by ^{31}P NMR (Webb, 1982), using the Bruker 360-MHz spectrometer (operating at 145.7 MHz) at the University of Pennsylvania School of Medicine NMR facility (Figure 1).

Single Turnover Experiment. A solution containing PPase (42 mg, specific activity 350 μmol of PP_i mg^{-1} min^{-1}), 0.1 M KCl, 0.1 M Tris (pH 7), and 1.75 mM $CdCl_2$ in a total volume of 0.8 mL was lyophilized and redissolved in 0.35 mL of $H_2{}^{18}O$. Separately, a solution of 90 mM Na_4[^{32}P]PP_i (45 μL, 7 mCi/mmol) was lyophilized and redissolved in 0.050 mL of $H_2{}^{18}O$. The two solutions were combined at 0 °C. Aliquots (4 μL) were removed at 8 and 16 min, and their [^{32}P]P_i content was determined by the molybdate–isobutyl alcohol method (Springs et al., 1981). The remaining reaction solution was quenched after 20 min by adding 0.2 mL of cold 1 M $HClO_4$. Following centrifugation to remove precipitated protein, the supernatant was adjusted to pH 8 with 5 N KOH (46 μL). Excess 2 M KCl (53 μL) was added, and the resulting $KClO_4$ precipitate was removed by centrifugation. The supernatant was diluted with 25 mL of cold water and loaded onto a Dowex 1 column (Cl^- form, 3 × 0.5 cm). P_i was eluted with 30 mM HCl, leaving PP_i on the column. The pooled fractions were brought to pH 8 with Tris base, lyophilized, and dissolved in 1.5 mL of 50% D_2O in water containing 50 mM EDTA for ^{31}P NMR analysis (Figure 2A). The chemical shift difference induced by ^{18}O substitution (Cohn & Hu, 1978) was used to determine the ^{18}O content of the P_i. As a control for this experiment, the above procedure was repeated exactly, but PPase was omitted and $H_2{}^{16}O$ replaced $H_2{}^{18}O$ (Figure 2B).

Results

PPase catalyzes hydrolysis of ATPγS and of inorganic thiophosphate. A variety of divalent metal ions alone or in combination were found to confer ATPγS hydrolase activity on PPase, as shown in Table I. The following points may be made: (a) ATPγS hydrolase activity is both PPase and divalent metal ion dependent (experiment 1 and footnote *b*). (b) The key divalent metal ion for ATPγS hydrolase activity is Mn^{2+}. Mn^{2+} alone confers substantial relative activity (experiments 3 and 8), and although such activity can be enhanced on addition of either Zn^{2+} (experiment 10) or Co^{2+} (experiment 11), neither of these latter two metal ions alone confer appreciable activity (experiments 6 and 7). No activity is seen in the presence of Mg^{2+} (experiment 2), and this metal ion inhibits Mn^{2+}-dependent activity (experiments 8 and 9).

Table I: Relative Rate Constants for PPase Catalysis of ATPγS and SP_i Hydrolysis[a]

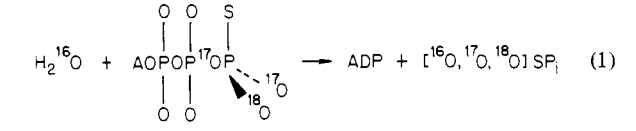
		divalent metal ion concn (mM)					relative[b] rate
expt	substrate	Mg^{2+}[c]	Mn^{2+}	Zn^{2+}	Co^{2+}	pH	constant
1	ATPγS					7.5	<0.01
2	ATPγS	1.3				7.5	<0.01
3	ATPγS		0.65			7.5	0.16
4	ATPγS		1.3			7.5	0.09
5	ATPγS		2.6			7.5	0.07
6	ATPγS				1.3	7.5	0.014
7	ATPγS			1.3		6.5	0.02
8	ATPγS[c]		0.65			6.7	0.6
9	ATPγS	0.65	0.65			6.7	0.3
10	ATPγS		0.65	0.65		6.7	0.7
11	ATPγS		0.65		0.65	6.7	1.0
12	SP_i		0.5	0.5		7.5	0.11
13	SP_i		0.5	0.5		6.7	0.09

[a] All reactions were run at 37 °C in 100 mM Tris buffer (pH 7.5) or 100 mM Mes (pH 6.7) buffer adjusted to the appropriate pH with HCl at a PPase concentration of 2 mg/mL. For experiments 1–11, ATPγS concentration was 1.3 mM. For experiments 12 and 13, SP_i concentration was 2.0 mM. In all reactions, DTT was added at a concentration equimolar with substrate except as otherwise indicated. [b] All rate constants are based on single-point kinetics (1–6 h) and are corrected for background hydrolysis in the absence of PPase. Such hydrolysis showed some variation as a function of added divalent metal ion(s) but never exceeded a relative value of 0.04. The relative value of 1.0 corresponds to a first-order rate constant 3.1 × 10^{-4} s^{-1}. [c] In this reaction, DTT was present in a 5-fold molar excess over ATPγS.

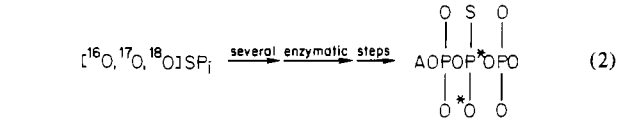
(c) ATPγS activity in the presence of Mn^{2+} is higher at pH 6.7 than at pH 7.5 (experiments 3 and 8).

PPase catalyzes not only PP_i and ATP hydrolysis but also H_2O–P_i oxygen exchange. It is clear from experiments 12 and 13 that PPase catalyzes as well hydrolysis of SP_i to P_i, which is presumably analogous to the latter reaction. Since isolation of SP_i is essential for the stereochemical experiments, it was important to select conditions for PPase catalysis of ATPγS hydrolysis that maximize SP_i recovery. It is clear from Table I (experiment 3 vs. experiment 8, experiment 12 vs. experiment 13) that pH 6.7 is superior to pH 7.5 for this purpose. Other experiments also showed that higher recoveries of SP_i were obtained in the presence of Mn^{2+} and Zn^{2+} than in the presence of Mn^{2+} and Co^{2+}. Accordingly, these two metal ions were chosen as the divalent metal ion cofactors for the stereochemical study.

PPase Catalysis of ATPγS Hydrolysis Proceeds with Inversion. The SP_i released following PPase catalysis of the hydrolysis of [β,γ-^{17}O,γ-^{17}O,^{18}O]ATPγS (eq 1) was converted



stereospecifically to the β-phosphoryl group of ATPβS with loss of one oxygen (eq 2; asterisks in this equation represent possible positions of isotopic labeling), exactly as described



previously (Webb, 1982). A ^{31}P NMR spectrum of the ATPβS prepared is shown in Figure 1. As has been previously discussed, the peak containing a single ^{18}O in the β, γ bridging position is derived from [^{16}O,^{17}O,^{18}O]SP_i in the *R* configuration (corresponding to inversion), and the peak containing a single

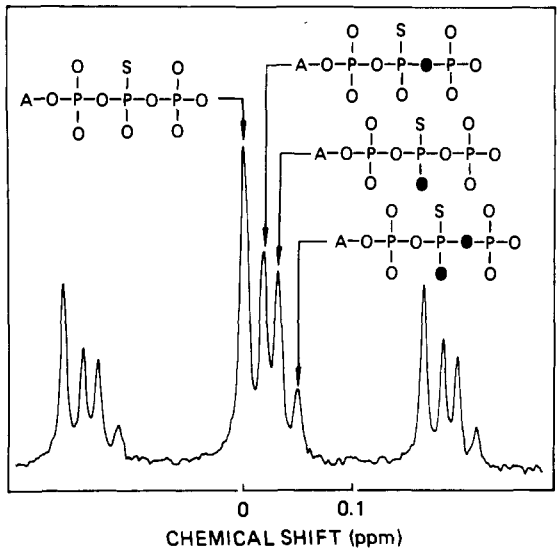
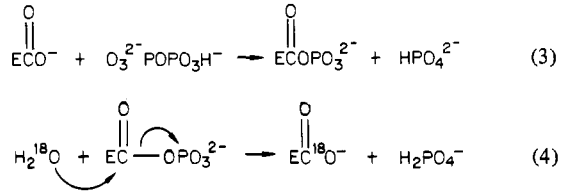


FIGURE 1: ^{31}P NMR spectrum of the β-phosphorus of ATPβS. The ATPβS was derived from SP_i produced on PPase-catalyzed hydrolysis of [β,γ-^{17}O,γ-^{17}O,^{18}O]ATPγS. The solid circles correspond to ^{18}O.

^{18}O in the β-phosphoryl position is derived from [^{16}O,^{17}O,^{18}O]SP_i in the *S* configuration (corresponding to retention). Due to quadrupolar broadening, β-phosphoryl groups containing ^{17}O oxygens are not visible in the spectrum.

On the basis of the isotopic composition of the labeled ATPγS, one can calculate the expected relative intensities of the four peaks labeled in Figure 1, assuming reaction 1 proceeds with complete inversion. This calculation is presented in Table II, row 1, and deviates markedly from the observed ratio (row 4). Such deviation results from the reaction sequence incorporating SP_i into ATPβS (Webb, 1982). In particular, nonenzymatic hydrolysis of glycerate 1-thiophosphate 3-phosphate competes with its enzymatic formation from SP_i and glyceraldehyde 3-phosphate (via glyceraldehyde-phosphate dehydrogenase) and its enzymatic incorporation into ATPγS (via phosphoglycerate kinase). Hydrolysis of glycerate 1-thiophosphate 3-phosphate leads to a re-formation of SP_i and is accompanied both by a dilution of ^{17}O and ^{18}O contents in SP_i and by racemization of SP_i. The extent of such hydrolysis can be directly estimated from the difference between the calculated (row 1) and the observed (row 4) intensities of the unlabeled peak. When such hydrolysis is taken into account, the corrected calculated values for peak intensities, assuming that ATPγS hydrolysis proceeds with inversion (row 2), agree very well with the observed values. We therefore conclude that PPase catalysis of ATPγS hydrolysis proceeds with inversion.

Oxygen Incorporated in the Enzyme-Catalyzed Hydrolysis of PP_i to Two Molecules of P_i Appears To Come Directly from Solvent Water. The stereochemical result presented above is strong evidence for a single nucleophilic attack on phosphorus during PPase catalysis. However, PPase catalysis could still involve an acyl phosphate intermediate, with the acyl group corresponding to either a glutamyl or an aspartyl side chain, if hydrolysis of the acyl phosphate proceeded via C–O cleavage (eq 3 and 4). In the absence of intermedi-



(3)

(4)

Table II: Calculated and Observed Peak Intensities for the ^{31}P NMR Spectrum of the β-Phosphorus of ATPβS Derived from SP_i Produced on PPase-Catalyzed Hydrolysis of [β,γ-^{17}O,γ-^{17}O,^{18}O]ATPγS

	relative peak intensities			
	unlabeled	^{18}O, bridging	^{18}O, nonbridging	$^{18}O_2$
calcd from known isotopic composition of ATPγS and assuming inversion[a]	0.16	0.45	0.26	0.13
calcd assuming inversion and corrected for isotope loss and racemization[b]	0.37	0.31	0.25	0.07
calcd assuming retention and corrected for isotope loss and racemization	0.37	0.25	0.31	0.07
obsd[c]	0.37	0.30	0.25	0.08

[a] These isotopic compositions were calculated from the ^{31}P NMR spectrum of [β,γ-^{17}O,γ-^{17}O,^{18}O]ATPγS, 95% for ^{18}O and 44% for ^{17}O. The ^{17}O positions also contain 29% ^{18}O [see Webb (1982)]. [b] The observed intensity of the unlabeled peak allows calculation of a 24% loss of isotope and an 8% racemization during the conversion of SP_i to ATPβS (Webb et al., 1980; Webb & Eccleston, 1981; Webb, 1982). [c] As determined from Figure 1.

ate–water exchange, this possibility requires that during the first turnover of the enzyme oxygen from solvent would not be incorporated into P_i. Measurement of the isotopic content of the P_i produced when PP_i is hydrolyzed by PPase in $H_2{}^{18}O$ under single turnover conditions provides a test of the mechanism represented by reactions 3 and 4. During single turnover, 2 mol of $^{16}O_4P$ should be produced per mol of PP_i hydrolyzed. If, on the contrary, the oxygen incorporated into P_i comes directly from solvent, then 1 mol each of $^{16}O_4P$ and $^{18}O^{16}O_3P$ should be produced per mol of PP_i hydrolyzed.

The results of such an experiment are presented in Figure 2A. Cd^{2+} was chosen as a divalent metal ion cofactor first, because the relatively low activity it confers made quenching the reaction after approximately a single turnover quite simple and, second, because there is no intermediate–water exchange in the presence of this metal ion (Welsh et al., 1983a). As can be seen, the peak for $^{16}O_4P$ ($^{18}O_0$) has an integrated intensity about one-third higher than that for the $^{18}O^{16}O_3P$ ($^{18}O_1$) peak. However, if the $^{18}O_0$ formed nonenzymatically during the workup procedure is taken into account (Figure 2B) and subtracted from the $^{18}O_0$ peak in Figure 2A, then the ratio of $^{18}O_0$:$^{18}O_1$ formed enzymatically is calculated as 0.8:1.0. As this ratio is indistinguishable from 1:1 within the precision of our experiment, we conclude that the oxygen incorporated into P_i on PPase-catalyzed PP_i hydrolysis comes directly from water.

It must be noted that this conclusion is subject to the caveat that if the putative active-site carboxylate oxygens undergo exchange with solvent water at a rate which is rapid compared to that of enzyme-catalyzed PP_i hydrolysis, then we could obtain the present result (Figure 2A) without requiring direct attack by solvent water on PP_i. We considered a sufficiently rapid exchange, however, to be extremely unlikely. The known pH and temperature dependences of acetic acid–water oxygen exchange (Llewellyn & O'Connor, 1964) allow estimation of a rate constant for carboxyl side chain–water exchange at pH 7 and 0 °C of $\leq 2.5 \times 10^{-10}$ min^{-1}. By contrast, for such exchange to be important for our results, a half-life of about

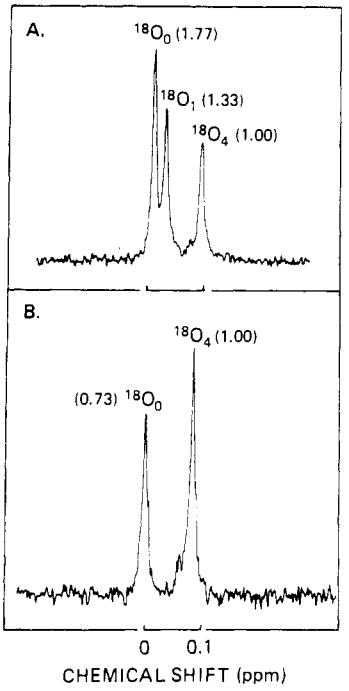


FIGURE 2: ^{31}P NMR spectra of (A) P_i produced during a single turnover by Cd^{2+}-PPase of PP_i hydrolysis in $H_2{}^{18}O$, with subsequent quench and purification in $H_2{}^{16}O$, and of (B) P_i produced in the absence of PPase when a sample of PP_i is subjected to the same quench and purification procedure as in (A). In both (A) and (B), an identical amount (0.9 μmol) of $P^{18}O_4$ was added as an internal standard. Numbers in parentheses represent integrated peak intensities relative to that for the $P^{18}O_4$ standard.

2 min, corresponding to a rate constant of 0.3 min^{-1}, would be required. The active site of PPase would thus have to be so structured as to cause a $\geq 10^9$ catalysis of water–carboxylate oxygen exchange, which would be remarkable considering that such a reaction is a virtual one not directly relevant to the catalytic function of PPase.

Discussion

In the presence of Mg^{2+}, the divalent metal ion conferring the highest enzymatic activity as cofactor, PPase is almost totally specific for PP_i as substrate, but in the presence of divalent ions such as Zn^{2+} or Mn^{2+}, other pyrophosphates such as ATP, tripolyphosphate, and seryl pyrophosphate are also reasonable substrates (Cooperman, 1982). As pointed out above, Mn^{2+} appears to be uniquely effective as a divalent metal ion cofactor for ATPγS hydrolysis.

Webb (1982) has made the point that ATPases using ATPγS as a substrate in place of ATP generally catalyze thiophosphoryl group transfer 10^2–10^3 times more slowly than they catalyzed phosphoryl transfer. This same range is appropriate for PPase catalysis of ATPγS hydrolysis. In the presence of Mn^{2+} and Co^{2+} (Table I, experiment 11), such hydrolysis proceeded at about 0.1% the rate found for PPase catalysis of ATP hydrolysis in the presence of Zn^{2+}, the divalent metal ion conferring maximal ATPase activity (experiment not shown).

Although the finding that overall phosphoryl transfer proceeds with inversion is evidence for single-step direct phosphoryl transfer, as described in the introduction, Mehdi & Gerlt (1982) have pointed out that reactions 3 and 4 described an enzymatic reaction with a phosphorylated intermediate that also proceeds with overall inversion at phosphorus. Such C–O cleavage of an acyl phosphate intermediate is perfectly plausible chemically. Indeed, Klinman & Samuel (1971) have shown that divalent metal ion catalyzed hydrolysis of acetyl phosphate by hydroxide ion attack proceeds exclusively via C–O cleavage. The possibility that C–O cleavage occurs during PPase catalysis was tested by the single turnover experiment (Figure 2); the results clearly show an absence of such cleavage if one makes the reasonable assumption that no extraordinarily rapid water–carboxylate oxygen exchange occurs at the active site. Thus, the overall inversion we observe on ATPγS hydrolysis is strong evidence against a phosphorylated enzyme intermediate in PPase catalysis and for a single-step, direct transfer mechanism.

In concluding this paper, it is necessary to point out that the two major experiments we report have both been carried out with poor substrates. PPase catalysis of ATPγS hydrolysis proceeds at only 0.1% the rate of PPase catalysis of ATP hydrolysis (in the presence of Zn^{2+}) and only 10^{-5} the rate of PPase catalysis of PP_i hydrolysis (in the presence of Mg^{2+}) (Cooperman, 1982). Cd^{2+}-PPase, used in the single turnover experiment, has only about 10^{-4} as much activity as does Mg^{2+}-PPase (Welsh et al., 1983b). Thus, using our present results to reach general conclusions about the PPase chemical mechanism requires the assumption that this mechanism is essentially the same for both good and poor substrates. One important reason for believing this is true is that the turnover number for Mg^{2+}-PPase catalysis of PP_i hydrolysis is about 10^{10} times larger than the first-order rate constant for uncatalyzed PP_i hydrolysis in water, so that PPase is a potent catalyst even for the poor substrates employed in this study. Further, a detailed study of Cd^{2+}-PPase activity has revealed many similarities between the Cd^{2+}- and Mg^{2+}-activated enzymes (Welsh et al., 1983a).

Registry No. [β,γ-^{17}O,γ-^{17}O,^{18}O]ATPγS (S_p isomer), 88454-60-8; PPase, 9024-82-2; pyrophosphate, 2466-09-3.

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Nickel Tetrapyrrole Cofactor F_{430}: Comparison of the Forms Bound to Methyl Coenzyme M Reductase and Protein Free in Cells of *Methanobacterium thermoautotrophicum* ΔH†

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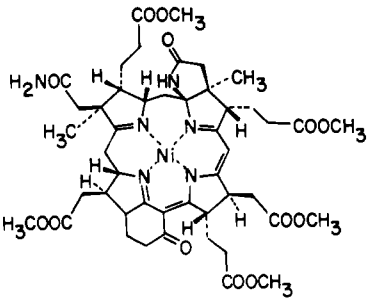
ABSTRACT: The nickel tetrapyrrole cofactor F_{430} occurs in two intracellular forms in *Methanobacterium thermoautotrophicum* ΔH. One form is bound to the methyl coenzyme M reductase as previously described [Ellefson, W. L., Whitman, W. B., & Wolfe, R. S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3707–3710]. A simple high-yield purification of this enzyme is described. A second form, protein-free F_{430}, was purified by using ion-exchange, gel filtration, and reverse-phase chromatography. The protein-bound F_{430} was released from the pure enzyme by using gentle extraction procedures, and the two forms were compared. The extracted and protein-free species were identical by ultraviolet–visible spectroscopy, reverse-phase high-performance liquid chromatography elution position, coenzyme M analysis, and mass spectrometry. Our data suggest that the complete F_{430} molecule is the free pentaacid form (m/z = 905) of the F_{430} structure proposed by Pfaltz et al. [Pfaltz, A., Jaun, B., Fassler, A., Eschenmoser, A., Jaenchen, R., Gilles, H. H., Diekert, G., & Thauer, R. K. (1982) *Helv. Chim. Acta 65*, 828–865]. F_{430} does not possess covalently bound coenzyme M or lumazine derivatives.

In 1978, Gunsalus & Wolfe (1978) first reported the isolation of a yellow, nonfluorescent chromophore from heat-treated extracts of *Methanobacterium thermoautotrophicum* ΔH. This compound, named factor F_{430}[1] because of its absorbance maximum at 430 nm, has since been observed in all methanogens examined (Diekert et al., 1981). F_{430} was recently shown to be the chromophore in methyl-*S*-CoM reductase (Ellefson et al., 1982). This enzyme catalyzes the last step in methanogenesis, the two-electron reductive cleavage of methyl-*S*-CoM (methyl mercaptoethanesulfonate) to methane and HSCoM (coenzyme M, mercaptoethanesulfonate) (Ellefson & Wolfe, 1980; Nagle & Wolfe, 1983a). Enzyme-bound

$$CH_3S\wedge SO_3^- + 2H^+ + 2e^- \longrightarrow CH_4 + HS\wedge SO_3^-$$

factor F_{430} exhibited an absorbance peak at 422 nm and a 445-nm shoulder (Ellefson et al., 1982). Boiling methanol extraction of the enzyme released the chromophore as the 430-nm-absorbing species with a stoichiometry of 1.75 mol of F_{430} bound per 1 mol of protein (M_r 300 000) (Ellefson et al., 1982). The role of F_{430} in catalysis by the methyl-*S*-CoM reductase has not been elucidated.

F_{430} is the only known biological example of a nickel–tetrapyrrole complex (Diekert et al., 1980a,b; Pfaltz et al., 1982). The structure for the methanolysis product of F_{430} has recently been established by Pfaltz et al. (1982). As shown below (compound I), the chromophore is a highly reduced, tetrahydro derivative of the corphin system—a structural hybrid of porphyrins and corrins. The ligand skeleton is that



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of a uroporphinoid (type III) with an additional carbocyclic ring. Although the macrocyclic ring structure is now fully established in $HClO_4$-extracted F_{430} (Pfaltz et al., 1982), it has been claimed by others that there are additional components associated with the native cofactor in cells. Vogels and co-workers have purified a series of incompletely defined F_{430} species differing in redox state or associated components. They suggested that native F_{430} includes bound HSCoM (Keltjens et al., 1982), a lumazine derivative (Keltjens et al., 1983a), and perhaps other substituents (Keltjens et al., 1983b). Furthermore, in collaboration with Wolfe and colleagues, they found HSCoM was associated in stoichiometric amounts with extracted F_{430} from pure methyl-*S*-CoM reductase (Keltjens et al., 1982).

In this paper, we demonstrate that for *M. thermoautotrophicum* ΔH grown on 1 μM Ni, F_{430} exists in two pools. These pools include the enzyme-bound species discussed by Ellefson et al. (1982) and a previously unreported protein-free form.

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[1] Abbreviations: F_{430}, nickel tetrapyrrole cofactor of methanogenic bacteria; HSCoM, 2-mercaptoethanesulfonic acid (coenzyme M); DE52, diethylaminoethylcellulose; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; KP_i, potassium phosphate.

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