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# Nonequilibrium Isotope Exchange Reveals a Catalytically Significant Enzyme−Phosphate Complex in the ATP Hydrolysis Pathway of the AAA$^+$ ATPase Magnesium Chelatase

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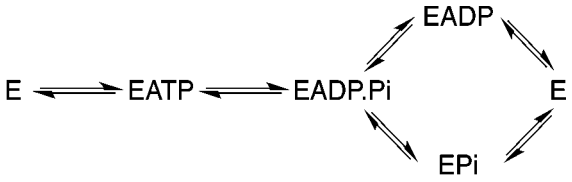
Supporting Information

**ABSTRACT:** Magnesium chelatase is an AAA$^+$ ATPase that catalyzes the first committed step in chlorophyll biosynthesis. Using nonequilibrium isotope exchange, we show that the ATP hydrolysis reaction proceeds via an enzyme−phosphate complex. Exchange from radiolabeled phosphate to ATP was not observed, offering no support for an enzyme−ADP complex.

The AAA$^+$ superfamily of enzymes couple ATP hydrolysis to an exceptionally broad range of biological activities.[1−4] These enzymes hydrolyze ATP, driving a series of conformational changes that then modify biological activity. Consistent with this view, many enzymes in the superfamily adopt different structures with ATP bound, ADP bound, and nucleotide free.[5−8] These structures are often interpreted as reflecting the different conformations adopted during an ATP hydrolysis cycle.

With two products, ADP and phosphate ($P_i$), enzyme-catalyzed hydrolysis of ATP could conceivably involve enzyme−ADP (EADP) or enzyme−phosphate ($EP_i$) complexes (Scheme 1). AAA$^+$ ATPases could prefer either pathway.

**Scheme 1. Random Release of Products from Enzyme-Catalyzed ATP Hydrolysis**[a]

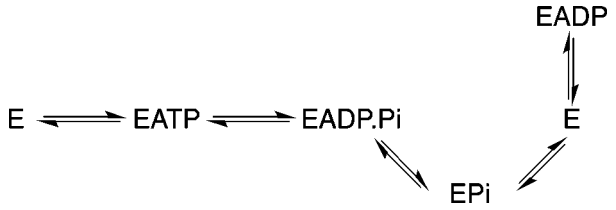



[a]$Mg^{2+}$ and $H^+$ omitted for the sake of clarity.

We present evidence that a reactive enzyme−phosphate complex is formed by the AAA$^+$ ATPase magnesium chelatase.

Structural and biochemical evidence demonstrates that members of the AAA$^+$ superfamily can form both EADP and $EP_i$ complexes.[5−9] While it is tempting to conclude that a structurally characterized enzyme−product complex is on the reaction pathway, the same species could arise as a dead-end inhibitor complex (Scheme 2). Additional approaches are needed to demonstrate that a species is catalytically relevant. We have used a simple, effective, and well-established method to show that magnesium chelatase, a member of the AAA$^+$ superfamily, has a kinetic mechanism that involves an enzyme−phosphate complex.

**Scheme 2. Ordered Release of Products from an Enzyme-Catalyzed ATP Hydrolysis Proceeding via an Enzyme−Phosphate Complex**[a]




[a]ADP acts as a dead-end inhibitor. $Mg^{2+}$ and $H^+$ omitted for the sake of clarity.

Magnesium chelatase is a well-characterized member of the family that catalyzes the first committed step in chlorophyll biosynthesis.[10−16] The chelatase contains a porphyrin binding subunit (ChlH) and two types of AAA$^+$ ATPase subunits, the active ChlI and the inactive ChlD.[13,14] ChlI and ChlD form a complex with reduced ATPase activity (ChlID).[14] All three subunits (ChlIDH) are needed to couple ATP hydrolysis to metalloporphyrin synthesis. We demonstrate that magnesium chelatase produces an $EP_i$ complex on the ATP hydrolysis pathway by observing nonequilibrium isotope exchange between radiolabeled ADP and substrate.

In these experiments, a small amount of radiolabeled product ([$\alpha$-$^{32}$P]ADP or [$^{32}$P]$P_i$) was added to conventional magnesium chelatase reaction mixtures with the substrates required for ATP hydrolysis and metal ion chelation (ATP, $MgCl_2$, and porphyrin). All reaction mixtures also included coupling enzymes to remove the nonradiolabeled product of ATP hydrolysis (pyruvate kinase for ADP and purine nucleoside phosphorylase for $P_i$). The rate of synthesis of radiolabeled ATP was then followed. Under these conditions, radiolabeled ATP can appear only if an enzyme−product complex reacts with the radiolabeled species (Scheme 3). Therefore, exchange of [$\alpha$-$^{32}$P]ADP with ATP would be evidence of the existence of an $EP_i$ complex, and exchange of [$^{32}$P]$P_i$ with ATP would be evidence of an EADP complex.

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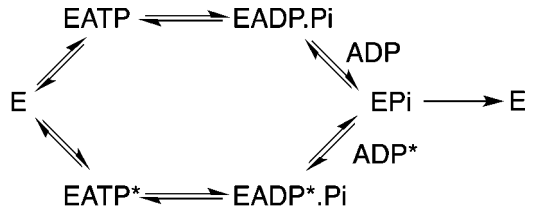
**Scheme 3. Ordered Product Release in the Presence of an Auxiliary Enzyme To Prevent $P_i$ Binding[a]**




[a]Radiolabeled ADP (ADP*) can produce only radiolabeled ATP through reaction with an $EP_i$ complex as $P_i$ in solution is removed by reaction with 7-methylguanosine catalyzed by purine nucleoside phosphorylase.

We observe exchange between [$\alpha$-$^{32}$P]ADP and ATP providing direct evidence of a significant enzyme−phosphate species. Our analogous experiments using [$^{32}$P]$P_i$ show no exchange with ATP and provide no evidence of a catalytically significant EADP complex (Table 1).

**Table 1. Observed Rates of Exchange of the $^{32}$P-Labeled Product with [$^{32}$P]ATP Catalyzed by Magnesium Chelatase or Component Proteins[a]**

| | $v_i$/[ChlI] (min$^{-1}$) | |
|---|---|---|
| protein | [$\alpha$-$^{32}$P]ADP → [$\alpha$-$^{32}$P ]ATP | $^{32}P_i$ → [$\gamma$-$^{32}$P]ATP |
| ChlI | 0.52 ± 0.09 | not quantifiable |
| ChlD | not observed | not observed |
| ChlH | not observed | not observed |
| ChlID | 0.19 ± 0.04 | not observed |
| ChlIDH | 0.96 ± 0.15 | not observed |
| ChlIDH and porphyrin ($D_{IX}$) | 1.44 ± 0.05 | not observed |

[a]The chelatase (ChlIDH) contains a porphyrin binding subunit without ATPase activity (ChlH) and a complex of AAA$^+$ subunits (ChlID). ChlI is the ATPase component of the chelatase, while isolated ChlD has no ATPase activity.

We can readily observe exchange of [$\alpha$-$^{32}$P]ADP with ATP (Table 1). The intact enzyme catalyzes this reaction in the presence of all substrates, demonstrating that an $EP_i$ complex is on the reaction pathway for coupled ATP hydrolysis. Exchange is also observed in the uncoupled ATPase reaction in the absence of porphyrin. The isolated ATPase components, ChlI, and the ChlID complex also catalyze exchange from [$\alpha$-$^{32}$P]ADP. So all known ATPase components of this enzyme show a [$\alpha$-$^{32}$P]ADP to ATP exchange activity.

Our results have clearly demonstrated the existence of a catalytically significant $EP_i$ complex in the ATPase reaction of magnesium chelatase. Provided that the $EP_i$ complex is capable of reacting with ADP, both covalent and noncovalent complexes can show isotope exchange.[17] Previous attempts to isolate a covalent $EP_i$ complex of magnesium chelatase have been unsuccessful.[18] The observations reported here can be explained perfectly satisfactorily without invoking a covalent phosphoenzyme.

Similar exchange reactions of magnesium chelatase subunits with radiolabeled ADP have been previously described.[18,19] In these experiments, no coupling enzymes were present to remove the phosphate product. As a result, the origin of the observed exchanges was ambiguous, and they could have arisen from the reaction of the chelatase with $P_i$ and radiolabeled ADP from solution. Our use of purine nucleotide phosphorylase to remove $P_i$ from solution reveals the role of an $EP_i$ complex in the reaction.

Formation of ATP from ADP could arise from contamination with the highly active *Escherichia coli* adenylate kinase. The adenylate kinase reaction produces ATP and AMP from two ADP molecules; we observe no AMP formation. Adenylate kinase is inhibited by the bisubstrate analogue, $P^1$,$P^5$-di(adenosine-5′) pentaphosphate ($AP_5A$); we observe no inhibition of the exchange reaction upon addition of 100 $\mu$M $AP_5A$. We conclude that the exchange observed from [$\alpha$-$^{32}$P]ADP to ATP does not arise from contamination with adenylate kinase.

In contrast to the reaction with [$\alpha$-$^{32}$P]ADP, the intact chelatase does not catalyze exchange of [$^{32}$P]$P_i$ with ATP (Table 1). An unquantifiably small amount of exchange (<1%) was seen in the presence of the AAA$^+$ ATPase subunit ChlI. This observation suggests that a minor reversible reaction pathway is accessible to this subunit via an EADP complex.

Our work provides no evidence of a catalytically significant EADP complex. The complex may exist but be undetected by this exchange methodology. Two plausible conditions could prevent exchange from being observed. First, exchange experiments can fail to detect a complex when binding of the partner product is too weak to allow the reaction to proceed at an appreciable rate. We do not observe any inhibition of the reaction by $P_i$ (up to 10 mM), demonstrating that binding of $P_i$ to the enzyme is weak (data not shown). Second, an irreversible step before the formation of EADP would prevent formation of labeled ATP via this intermediate. Structurally, this could be envisaged as a large conformational change at the subunit interface moving the arginine finger, a putative phosphate binding residue, away from the ADP-binding portion of the active site, disrupting the $\gamma$-$P_i$ subsite. Such large conformational changes have often been proposed in ATPase cycles of AAA$^+$ enzymes and are consistent with the observation of both tight and loose subunit interfaces in the AAA$^+$ component of *Rhodobacter sphaeroides* magnesium chelatase.[10]

We have clearly demonstrated here that the ATPase pathway of magnesium chelatase proceeds via an enzyme−phosphate complex. While we have no evidence to support the existence of an enzyme−ADP complex, we are reluctant to rule it out as a low affinity for phosphate could prevent detection of this species. The demonstration that ATP hydrolysis catalyzed by a member of the AAA$^+$ superfamily can proceed through an enzyme−phosphate complex expands our understanding of the potential routes to ATP hydrolysis in this vast group of biologically important proteins.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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