Kinetic and Stability Properties of Penicillium chrysogenum ATP Sulfurylase Missing the C-terminal Regulatory Domain*

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Eissa Hanna‡§, Kit Fai Ng§¶, Ian J. MacRae‡, Christopher J. Bley‡, Andrew J. Fisher¶, and Irwin H. Segel†**

From the ‡Section of Molecular and Cellular Biology and ¶Department of Chemistry, University of California, Davis, California 95616

ATP sulfurylase from Penicillium chrysogenum is a homohexameric enzyme that is subject toallosteric inhibition by 3'-phosphoadenosine 5'-phosphosulfate. In contrast to the wild type enzyme, recombinant ATP sulfurylase lacking the C-terminal allosteric domain was monomeric and noncooperative. All kcat values were decreased (the adenosine 5'-phosphosulfate (adenylylsulfate) (APS) synthesis reaction to 17% of the wild type value). Additionally, the Michaelis constants for MgATP and sulfate (or molybdate), the dissociation constant of EAPS, and the monovalent oxynion dissociation constants of dead end E-MgATP-oxynion complexes were all increased. APS release (the k5 step) was rate-limiting in the wild type enzyme. Without the C-terminal domain, the composite k5 step (isomerization of the central complex and MgPPi release) became rate-limiting. The cumulative results indicate that besides (a) serving as a receptor for the allosteric inhibitor, the C-terminal domain (b) stabilizes the hexameric structure and indirectly, individual subunits. Additionally, (c) the domain interacts with and perfects the catalytic site such that one or more steps following the formation of the binary E-MgATP and E-SO42- complexes and preceding the release of MgPPi are optimized. The more negative entropy of activation of the truncated enzyme for APS synthesis is consistent with a role of the C-terminal domain in promoting the effective orientation of MgATP and sulfate at the active site.

Most plants and microorganisms can use inorganic sulfate as their sole source of sulfur. Because sulfate is nonreactive at cellular temperatures and pH, the anion must first be “activated” in order to enter the mainstream of metabolism. Activation proceeds in two steps. These are catalyzed, in order, by the enzymes ATP sulfurylase (MgATP:sulfate adenyllyltransferase; EC 2.7.7.4) and adenosine 5'-phosphosulfate (APS)1 kinase (MgATP:APS 3'-phosphotransferase; EC 2.7.1.25). The sequential reactions produce the sulfonucleotides APS and 3'-phosphoadenosine 5'-phosphosulfate (PAPS).

\[
\text{MgATP} + SC\text{v}_\text{APS} \rightleftharpoons \text{MgPP} + \text{APS (ATP sulfurylase)}
\]

\[
\text{MgATP} + \text{APS} \rightleftharpoons \text{MgADP} + \text{PAPS (APS kinase)}
\]

REATIONS 1 AND 2

ATP sulfurylase from the filamentous fungus, Penicillium chrysogenum, is a homooligomer composed of six 63.7-kDa subunits (573 residues). PAPS, the APS kinase product, is an allosteric inhibitor (1, 2). This inhibition may be part of a sequential feedback process, considering that PAPS is a major branch point metabolite in filamentous fungi but not in other organisms. (PAPS enters into the cysteine biosynthetic pathway and is also used by filamentous fungi for the formation of choline-O-sulfate, a sulfur storage compound and/or osmoprotectant (3–6)).

P. chrysogenum ATP sulfurylase is organized as a dimer of triads (7–9). Each subunit is composed of three structurally distinct globular regions. Residues 1–170 compose a distinct N-terminal domain. Residues 171–395 compose the central catalytic domain. Several residues that have been shown to be essential for activity (10, 11) are located in this domain. Residues 331–389 form a small subdomain, called Domain III in the yeast structure (12, 13). The allosteric site is located in a C-terminal domain that is very similar to APS kinase in sequence (14) and structure (15, 16). However, this regulatory domain (residues 396–573) has no APS kinase activity because of modifications to the ATP P-loop (17) and the filling of the ATP binding region with protein side chain surrogates (e.g. Phe-548, which fills the space that would otherwise be occupied by the adenine ring of ATP). PAPS is believed to initiate the allosteric transition by disrupting a salt link between Arg-515 in the C-terminal domain of one subunit and Asp-111 in the N-terminal domain of a trans-triad subunit (9). In moving from the high substrate affinity R state to the low substrate affinity T state (18–20), the side chain of Arg-515 moves toward PAPS, the allosteric domain of each subunit pivots 27° relative to the catalytic and N-terminal domains, and the hexamer expands slightly in volume. The R to T transition is accompanied by the movement of a catalytic domain loop (residues 228–238, termed the active site switch), which flips “up” by 17 Å. Rotation about the interface between catalytic-allosteric domains provides the space for the switch to open. When the switch is in the closed position, Asp-234 interacts with and presumably modulates the charge on Arg-199 of the active site107QTRN200 sulfate/phosphosulfate motif (8, 9). We have suggested that the allosteric effector may not induce a totally new subunit conformation but rather may exploit the existing flexibility of the enzyme. Small switch movements may be part of the normal

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1 The abbreviations used are: APS, adenosine 5'-phosphosulfate (adenylylsulfate); PAPS, 3'-phosphoadenosine 5'-phosphosulfate (adenylylsulfate 3'-phosphate); MES, (2-N-morpholino)ethanesulfonic acid; E₃, Arrhenius activation energy; n_H, Hill coefficient.

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‡ An undergraduate honors research student.

§ An undergraduate honors research student.

** To whom correspondence should be addressed: Section of Molecular and Cellular Biology, University of California, One Shields Ave., Davis, CA 95616. Tel.: 530-752-3193; E-mail: ihsegel@ucdavis.edu.

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catalytic cycle, allowing each subunit to act independently with the “up” switch position corresponding to a low affinity (ligand release) conformation. A large switch movement in any one subunit may trigger the concerted allosteric transition.

In order to learn more about the allosteric transition and, particularly, more about the functional relationship of the C-terminal domain to the rest of the protein, we have examined the properties of recombinant \textit{P. chrysogenum} ATP sulfurylase missing residues 396–573. The results indicate that the C-terminal domain does more than just serve as a receptor for the allosteric inhibitor.

**MATERIALS AND METHODS**

**Coupling Enzymes and Chemicals—** Recombinant APS kinase and wild type ATP sulfurylase from \textit{P. chrysogenum} were expressed and purified as described earlier (17, 21). Yeast ATP sulfurylase was obtained from Sigma. PAPS synthesis was measured by the reverse ATP sulfurylase reaction after adding about 40 ml of chilled 40 mM Tris-Cl buffer, pH 8.0 (standard buffer), the same buffer, the protein was eluted at 2 ml/min with 500 ml of a 0 M gradient in 40 mM Tris-Cl, pH 8.0. The pooled fractions (50 ml) contained about 2 mM PAPS and 0.3 M NaCl. (PAPS concentrations were determined from double reciprocal plots and slope replots. The same data were analyzed by double reciprocal plots and the appropriate replots. Consequently, each kinetic constant for the APS synthesis and molybdysulfate reactions was determined from two or three different plots or curve fits. Activity in the ATP synthesis direction was analyzed by (a) double reciprocal plots of $I_v$ versus $I_P$ (PPi) at 500 μM (saturating) APS to obtain $K_{mP}$ and the $K_{iP}$ for the varied substrate. Replots of $I_v$ versus the cosubstrate concentration yielded the limiting $V_{max}$ and the $K_{iB}$ of the cosubstrate. The same data were analyzed by double reciprocal plots and the appropriate replots. The inhibitory effect of PAPS was determined by fitting the $I_v/I_0$ (fractional velocity) versus $I$ data to Equation 2, where $I_v$ represents the velocity in the presence of inhibitor, $I_0$ is the velocity at the same substrate concentrations in the absence of inhibitor, $Z$ is the starting value of $I_v/I_0$ at $I = 0$, $M$ is the maximum change in $I_v/I_0$ [II] is the inhibitor concentration, $K_i$ is the Hill coefficient, and $K$ is a constant. ($K$ is equivalent to $[I]_e^{m_0}$) Theoretically, $Z = 1.0$. If saturating PAPS drives the velocity to zero, $M$ would also equal 1.0.

$$
I_v = Z - M[I]_e^{m_0} \\

K_i = M[I]_e^{m_0} (Eq. 2)
$$

The limiting $K_i$ values for thiosulfate, monovalent oxanions, and APS and for PAPS binding to the catalytic site of the truncated enzyme were determined from double reciprocal plots and slope replots. The $K_i$ for PAPS binding to the truncated enzyme was also estimated from the $[I]_e^{lin}$ value of a $I_v/I_0$ versus [PAPS] plot at fixed subsaturating [MgATP] and [MoO$_4^-$].

$$
K_i = \frac{[I]_e^{lin}}{[I]_e^{lin}} (Eq. 3)
$$

DeltaGraph Pro 4.05c was used for all curve fits. Kinetic constants are reported as the mean determined from multiple experiments (or multiple plots/curve fits). The maximum variations were generally less than ±15% of the mean.
RESULTS

Native and Subunit Molecular Mass—The truncated enzyme was active and eluted from a Sephacryl S-100-HR column at a position partially overlapping (but slightly behind) that of fungal APS kinase (47.4 kDa). SDS gel electrophoresis yielded a subunit size of about 46 kDa. The theoretical subunit mass is 44 kDa, so it appears that the truncated enzyme is monomeric. Wild type fungal ATP sulfurylase is a hexamer organized as a dimer of triads in the shape of a flattened ellipsoid 134 Å in diameter × 73 Å (8). Each triad is stabilized by the head-to-tail interaction of a catalytic domain of one subunit with the C-terminal domain of the next. In addition, each C-terminal domain interacts across the triad interface with an N-terminal domain, a catalytic domain, and another C-terminal domain. Considering the many oligomer-stabilizing interactions of the C-terminal domain, it is not surprising that its absence results in a monomeric enzyme.

Stability of the Truncated Enzyme—Truncated P. chrysogenum ATP sulfurylase is much less heat-stable than the wild type enzyme. At temperatures above 30 °C, activity is lost in a first order fashion, as shown in Fig. 1A. To obtain a comparable series of inactivation curves for the wild type enzyme, a temperature range of 55–65 °C is required (Fig. 1B). For example, $t_{1/2}$ for inactivation of the truncated enzyme at 50 °C is about 0.3 min, whereas the wild type enzyme is stable for $>2$ h at that temperature. At 45 °C, the truncated enzyme has a $t_{1/2}$ of about 1.5 min. To obtain the same $t_{1/2}$ for the wild type enzyme, $T_m$ must be increased to about 62 °C. $E_a$ values for inactivation of the wild type and truncated enzymes are 107 and 62.3 kcal/mol, respectively (Fig. 1C). Clearly, hexamerization not only provides the means of propagating a concerted allosteric transition (18–20) but also confers thermal stability. This was not surprising, considering the multiple contacts made by each C-terminal domain of the wild type enzyme as noted above.

Sensitivity to Sulfhydryl and Arginine-targeted Reagents—Preincubation of the wild type enzyme (15 nM in active sites in 50 mM potassium phosphate buffer, pH 8.0, 30 °C) with 50 μM 5,5′-dithiobis(2-nitrobenzoic acid) or 150 μM N-ethylmaleimide resulted in a rapid decrease in activity subsequently measured at 50 μM MgATP and 100 μM MoO₄²⁻ (subsaturating substrate levels). The $t_{1/2}$ values for the two reagents were 20 and 45 s, respectively. This apparent inactivation (which is observed only at subsaturating substrate levels) is caused by increases in the $[S]_{0.5}$ values for both substrates concomitant with the induction of sigmoidal kinetics (28). Under the same preincubation conditions, the truncated enzyme retained >97% of its activity after 30 min. The results confirm that the effect of SH-reactive reagents on the wild type enzyme resulted solely from Cys-509 modification. Two other Cys residues (located in the N-terminal domain at positions 42 and 69) appear to be inaccessible to 5,5′-dithiobis(2-nitrobenzoic acid) and N-ethylmaleimide.

Both forms of the enzyme were irreversibly inactivated by 3 mM phenylglyoxal, an arginine-targeted reagent (29). (Activity was measured at 5 mM MgATP and 5 mM MoO₄²⁻.) Whereas there are many Arg residues in ATP sulfurylase, the loss of activity must result, at least in part, from modification of Arg-199 at the active site. (Substrates protect against inactivation (28).) The $t_{1/2}$ values were 5 min for both forms of the enzyme, indicating no major difference in the accessibility of essential Arg residues.

pH Profiles—At 1 mM MgATP and 5 mM molybdate, the molybdolysis reaction rates were nearly constant between pH 6.5 and 9.5 for both the wild type and the truncated enzyme (Fig. 2A). At subsaturating substrate concentrations, the wild type enzyme displayed what appeared to be a typical pH optimum curve, but the response of the truncated enzyme was still essentially flat (Fig. 2B). Consequently, the usual explanations for the pH effect were inapplicable; i.e., the decrease in activity at lower pH values displayed by the wild type enzyme cannot be attributed to a reduction in the level of the true substrate, MgATP²⁻ (which would be minimal anyway (30, 31)). Nor could it result from protonation of essential His residues (10, 11), which are believed to play a role in MgATP binding (32, 33). If these causes were relevant, the truncated enzyme would have behaved the same way. It is more likely that the decrease in activity exhibited by the wild type enzyme...
at low pH reflects its transition to the high substrate $K_m$ T state (21), a shift denied to the truncated enzyme. The Scatchard plots shown in Fig. 2 confirm that the wild type enzyme behaves cooperatively at pH 6.5, but the truncated enzyme displays normal hyperbolic behavior.

Inhibition by PAPS—At 0.5 mM MgATP and 0.1 mM molybdate, the wild type enzyme displayed a sigmoidal PAPS inhibition curve with $n_H = 2.6$ and a [PAPS]$_{0.5}$ of about 40 $\mu$M (Fig. 3). In contrast, neither the truncated $P. chrysogenum$ enzyme nor the yeast enzyme showed cooperative inhibition. The [PAPS]$_{0.5}$ values combined with the experimental substrate concentrations and the appropriate kinetic constants (see Equation 3, Table I, and Ref. 14) yielded estimates for the limiting $K_i$ values in the region of 60 and 180 $\mu$M for the truncated $P. chrysogenum$ and yeast enzymes, respectively. The inhibition of the noncooperative enzymes almost certainly results from PAPS binding to the APS subsite of the catalytic domain. PAPS is, after all, a nearly perfect structural analog of APS, and the crystal structures indicate that only small changes in the structure of the active site region are needed to accommodate the 3'-phospho group (although it is likely that the PAPS affinity of the wild type enzyme's active site is closer that to that of the hexameric yeast enzyme than to that of the truncated $P. chrysogenum$ enzyme). A more detailed analysis of the inhibition of the truncated enzyme (Fig. 4) yielded a limiting $K_i$ of 71 $\mu$M, considerably greater than the $K_{iq}$ of 0.5 $\mu$M for APS binding to its active site (see below), but still substantial. The binding of PAPS to the catalytic site as well as to the allosteric site of the wild type enzyme acts to decrease the degree of cooperativity that would otherwise be observed.

Comparative Activities of the Wild Type and Truncated Enzyme—Table I summarizes the limiting kinetic constants of wild type and truncated $P. chrysogenum$ ATP sulfurylase at pH 8.0, 30°C. It can be seen that eliminating the C-terminal domain reduces the $k_{cat}$ for molybdolysis and the reverse (ATP synthesis) reactions by about 40%. In contrast to this moderate effect, the $k_{cat}$ for the physiological APS synthesis reaction is decreased substantially from 10.8 to 1.8 s$^{-1}$. In addition, the Michaelis constants of the truncated enzyme for MgATP and sulfate (or molybdate) are an order of magnitude greater than those of the wild type enzyme. Truncation has no major effect on the affinity of the active site for MgATP and sulfate (i.e. $K_{ia}$ and $K_{ib}$ are essentially unaffected). The substrate interaction factor, $\alpha$, defined as $K_{ia}/K_{ia}$ (for MgATP) or $K_{ib}/K_{ib}$ (for sulfate) is 0.22 for the wild type enzyme but 2.5 for the truncated enzyme. The difference was equally pronounced for the molybdolysis reaction (0.03 versus 0.24). Because the kinetic mechanism is not completely rapid equilibrium, the Michaelis constants are not simple dissociation constants. Consequently, the increase in $K_m$ resulting from the loss of the C-terminal domain cannot be attributed solely to a decrease in the affinity of a binary E-S complex for the cosubstrate, although this could be a factor. A change in downstream rate constants, including those for catalysis and product release, may also play a role (see below).

The apparent equilibrium constant of the reaction obtained from the Haldane equation (Table I) differs by a factor of 2.7 for the two enzyme forms. But this is certainly a result of the cumulative error introduced when calculating $K_{eq}$ as the product of six experimental kinetic constants. ($K_{eq}$ should be the same regardless of the enzyme used to catalyze the reaction.)
The kinetics studies described below were performed to identify (or at least narrow the choice of) the step(s) that was affected by the loss of the C-terminal domain.

**Reactivity with Other Inorganic Substrates**—ATP sulfurylase is rather nonspecific for the inorganic substrate, accepting a variety of divalent oxyanions (Table II). Sulfate and fluorophosphate yield stable nucleotides that can be isolated (34, 35). Selenate yields APSe, which is unstable but has a lifetime long enough to be captured by APS kinase and phosphorylated to become PAPS (36–38). Tungstate and chromate, like molybdate, do not produce long-lived stable nucleotide products but rather become PAPSe (36–38). Tungstate and chromate, like molybdate, do not produce long-lived stable nucleotide products but rather are not entered into a reaction. The interaction factor for thiosulfate, MgATP

| Constant | Description | Wild type (63.7 kDa) | Truncated (44.4 kDa) |
|----------|-------------|----------------------|----------------------|
| $V_{\text{max}}$ | Maximal velocity of APS synthesis | 10.2 units $\times$ mg protein$^{-1}$ | 2.5 units $\times$ mg protein$^{-1}$ |
| $k_{\text{cat}}$ | Catalytic rate constant | 10.8 s$^{-1}$ | 1.8 s$^{-1}$ |
| $K_{E}$ | MgATP dissociation constant | 0.9 mM | 1.1 mM |
| $K_{E}$ | Michaelis constant for $SO_{4}^{2-}$ at saturating MgATP | 0.29 mM | 3.6 mM |
| $K_{E}$ | $E$SO$_4^{2-}$ dissociation constant | $1.4\ \text{mM}$ | $1.4\ \text{mM}$ |
| $K_{E}$ | Michaelis constant for MgATP at saturating $SO_{4}^{2-}$ | 0.21 mM | 2.6 mM |
| $h_{l}$ | Rate constant for catalysis and/or MgPP$\varepsilon$ release | 219 s$^{-1}$ | 1.9 s$^{-1}$ |
| $h_{s}$ | Rate constant for APS release | 11.4 s$^{-1}$ | 47.5 s$^{-1}$ |

**Molybdobolysis**

| Constant | Description | Wild type (63.7 kDa) | Truncated (44.4 kDa) |
|----------|-------------|----------------------|----------------------|
| $V_{\text{max}}$ | Maximal velocity of molybdobolysis | 22.8 units $\times$ mg protein$^{-1}$ | 18.5 units $\times$ mg protein$^{-1}$ |
| $k_{\text{cat}}$ | Catalytic rate constant | 24.4 s$^{-1}$ | 13.7 s$^{-1}$ |
| $K_{E}$ | MgATP dissociation constant | 0.9 mM | 1.1 mM |
| $K_{E}$ | Michaelis constant for $MoO_{4}^{2-}$ at saturating MgATP | 0.076 mM | 0.53 mM |
| $K_{E}$ | $E$MoO$_4^{2-}$ dissociation constant | 2.5 mM | 2.2 mM |
| $K_{E}$ | Michaelis constant for MgATP at saturating $MoO_{4}^{2-}$ | 0.027 mM | 0.27 mM |

**ATP synthesis**

| Constant | Description | Wild type (63.7 kDa) | Truncated (44.4 kDa) |
|----------|-------------|----------------------|----------------------|
| $V_{\text{max}}$ | Maximal velocity of ATP synthesis | 69 units $\times$ mg protein$^{-1}$ | 63 units $\times$ mg protein$^{-1}$ |
| $k_{\text{cat}}$ | Catalytic rate constant | 73.3 s$^{-1}$ | 46.6 s$^{-1}$ |
| $K_{E}$ | E-APS dissociation constant | 0.062 $\mu$M | 0.51 $\mu$M |
| $K_{E}$ | Michaelis constant for PP$\varepsilon$ at saturating APS | 9.2 $\mu$M | 25 $\mu$M |
| $K_{E}$ | Michaelis constant for APS at saturating PP$\varepsilon$ | 0.4 $\mu$M | 0.5 $\mu$M |
| $K_{e}$ | Equilibrium constant for the ATP synthesis reaction calculated from the Haldane equation | $3.2 \times 10^{-7}$ | $1.2 \times 10^{-7}$ |

The kinetic mechanism significantly; MgATP and sulfate (or molybdate or thiosulfate) bind randomly to the enzyme, monovalent oxyanions bind almost exclusively to their respective subsites equally well on both enzyme types, reflecting that MgATP alone and sulfate (or molybdate) alone bind to their respective subsites equally well on both enzyme types, the difference in $K_{E}$ suggests a role of the C-terminal domain in shaping the composite phosphosulfate subsite of the catalytic domain.

Pyrophosphate was noncompetitive with respect to MgATP and sulfate, but the truncated enzyme yielded double reciprocal plots of $1/v$ versus $1/[MgATP]$ at different fixed [MgPP$\varepsilon$] that intersected very close to the vertical axis. $K_{(p(app))}$ at 10 mM sulfate was 330 $\mu$M. With the wild type enzyme, the apparent inhibition constants for PP$\varepsilon$ are in the 0.6–3 $\mu$M region (39). The results suggest that E-APS, the enzyme species that normally binds MgPP$\varepsilon$, in the steady state, accounts for a very minor fraction of the truncated enzyme and that most of the inhibition seen at approximately $K_{(p(app))}$ levels resulted from MgPP$\varepsilon$, competing with MgATP for free E.

**Dead End Inhibition**—Inorganic thiosulfate was competitive with molybdate and noncompetitive with MgATP. The $K_{I}$ for thiosulfate dissociation from E$S_{2}O_{3}^{2-}$ was calculated to be 1.8 $\mu$M for the wild type enzyme and 2.2 $\mu$M for the truncated species, not a major difference between the two enzyme forms. The $K_{I}$ values for thiosulfate dissociation from E$MgATPS_{2}O_{3}^{2-}$ were 0.33 and 1.4 $\mu$M for the wild type and truncated forms, respectively. Thus, the wild type enzyme shows about the same degree of synergism between MgATP and thiosulfate ($\beta = 0.18$) as between MgATP and sulfate ($\alpha = 0.22$), although thiosulfate does not enter into a reaction. The interaction factor for thiosulfate, $\beta$, was 0.64, (about 3.5 times poorer) for the truncated enzyme.

Monovalent oxyanions, such as fluorosulfonate and chloride, were competitive with molybdate and uncompetitive with respect to MgATP. The $K_{I}$ for fluorosulfonate dissociation from E$S_{2}O_{3}^{2-}$ was calculated to be 1.8 $\mu$M for the wild type enzyme and 2.2 $\mu$M for the truncated species, not a major difference between the two enzyme forms. The $K_{I}$ values for fluorosulfonate dissociation from E$MgATPS_{2}O_{3}^{2-}$ were 0.33 and 1.4 $\mu$M for the wild type and truncated forms, respectively. Thus, the wild type enzyme shows about the same degree of synergism between MgATP and thiosulfate ($\beta = 0.18$) as between MgATP and sulfate ($\alpha = 0.22$), although thiosulfate does not enter into a reaction. The interaction factor for thiosulfate, $\beta$, was 0.64, (about 3.5 times poorer) for the truncated enzyme.
The nonreactivity of chlorate and nitrate is understandable. Although the oxygen atoms of these monovalent oxyanions fit nicely between Gln-197, Arg-199, and Ala-295 (main chain -NH) of the sulfate subsite, they do not have a fourth oxygen to point toward MgATP. In the case of fluorosulfonate (FSO₃⁻/H⁺) and perchlorate, (ClO₄⁻/H⁺), the fourth oxygen does not carry a sufficiently negative charge.

The reason for the inactivity of thiosulfate (SSO₃²⁻/H⁺) is not immediately obvious. If the 197QXRN₂₀₀₀ motif and Ala-295 preferentially hydrogen-bond to the three outer oxygen atoms (8), then the fourth outer atom that is oriented toward ATP would be the less electronegative sulfur. If, on the other hand, the outer sulfur atom binds to the main chain -NH of Ala-295, as in the crystal structure of the yeast enzyme (13), then another reason must be sought. Perhaps small differences in bond lengths make a difference. The S–S bond in thiosulfate is 2.1 Å long compared with 1.7 Å for the S–O bond. This difference may cause the trigonal plane described by three outer

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**Table II**

Reactivity of *P. chrysogenum* ATP sulfurylase with different inorganic substrates

The reactions were carried out in Tris-Cl, pH 8.0, 30 °C. The enzyme to which the ATP sulfurylase reaction was coupled is shown in parentheses next to the substrate. Ap₅A (135 μM) was included in assays of the selenate-dependent and arsenate-dependent reactions coupled to APS kinase.

| Substrate            | **k_{cat(app)″}** | **K_{m(app)″}** | **K_{m(app)″}** |
|----------------------|-------------------|-----------------|-----------------|
| **Wild type**        |                   |                 |                 |
| Truncated            |                   |                 |                 |
| **Wild type**        |                   |                 |                 |
| Truncated            |                   |                 |                 |
| SO₄²⁻ (APS kinase)   | 10.2              | 1.3             | 0.24            |
| FPO₃²⁻ (APS kinase)  | 2.3               | 0.7             | 0.09            |
| SeO₄²⁻ (APS kinase)  | 2.1               | 1.4             | 0.96            |
| SeO₄²⁻ (myokinase)   | 1.0               | 0.9             | 0.06            |
| HPO₄²⁻ (neither)     | 0.4               | —               | 3               |
| HAsO₄²⁻ (myokinase)  | 11.5              | 0.11            | 0.98            |
| HAsO₄²⁻ (APS kinase) | 0.5               | 0.08            | 1.25            |
| MoO₄²⁻ (myokinase)   | 23.9              | 16.8            | 0.01            |
| WO₄²⁻ (myokinase)    | 26.6              | 14.1            | 0.07            |
| CrO₄²⁻ (myokinase)   | 3.4               | 2.0             | 0.01            |

a * V_{max(app)″} and K_{m(app)″} were determined by extrapolating the 1/v versus 1/[oxyanion] double reciprocal plot at 5 mM MgATP. *k_{cat(app)″} was calculated from *V_{max(app)″} K_{m(app)″}; for Pᵢ, is more appropriately indicated as [Pᵢ]₀.5 because the primary velocity curve appeared to be slightly sigmoidal.

b *K_{m(app)″} was determined from a plot of 1/v versus 1/[MgATP] at 10 mM oxyanion substrate except for chromate, phosphate, and arsenate, which were maintained at 0.3, 20, and 20 mM, respectively.

c —, the truncated enzyme did not have measurable activity with Pᵢ as the inorganic substrate.

The nonreactivity of chloride and nitrate is understandable. Although the oxygen atoms of these monovalent oxyanions fit nicely between Gln-197, Arg-199, and Ala-295 (main chain -NH) of the sulfate subsite, they do not have a fourth oxygen to point toward MgATP. In the case of fluorosulfonate (FSO₃⁻/H⁺) and perchlorate, (ClO₄⁻/H⁺), the fourth oxygen does not carry a sufficiently negative charge.

The reason for the inactivity of thiosulfate (SSO₃²⁻) is not immediately obvious. If the 197QXRN₂₀₀₀ motif and Ala-295 preferentially hydrogen-bond to the three outer oxygen atoms (8), then the fourth outer atom that is oriented toward ATP would be the less electronegative sulfur. If, on the other hand, the outer sulfur atom binds to the main chain -NH of Ala-295, as in the crystal structure of the yeast enzyme (13), then another reason must be sought. Perhaps small differences in bond lengths make a difference. The S–S bond in thiosulfate is 2.1 Å long compared with 1.7 Å for the S–O bond. This difference may cause the trigonal plane described by three outer...
protein-interacting atoms of the inorganic substrate (O, O, and S) to tilt, moving the remaining negative oxygen away from the α-P of MgATP.

Rate Constants—As shown in the Appendix, the macrokinetic constants of the physiological reaction can be used to estimate $k_a$ (the rate constant for APS release) and then $k_5$ (a composite rate constant for MgPPi release and all preceding isomerizations of the central complex). The calculations indicate that APS release is almost completely rate-limiting in the wild type enzyme: $k_{cat,f} = 10.8$ s$^{-1}$, $k_a = 11.4$ s$^{-1}$, $k_5 = 219$ s$^{-1}$. Because $k_{cat,f}$ and $k_a$ are close, the calculation of $k_5$ probably has considerable error. But it is certainly greater than $k_a$. Also, the binding of APS to free $E$ is close to being diffusion-limited ($k_{-a}$ is calculated to be $1.8 \times 10^7$ M$^{-1}$ s$^{-1}$). The calculated $k_{-a}$ was $3.2 \times 10^7$ M$^{-1}$ s$^{-1}$. For the truncated enzyme, $k_{cat,f} = 1.8$ s$^{-1}$, $k_a = 47.5$ s$^{-1}$, $k_5 = 1.9$ s$^{-1}$, $k_{-a} = 2.3 \times 10^6$ M$^{-1}$ s$^{-1}$, and $k_{-a}$ is about $9.3 \times 10^5$ M$^{-1}$ s$^{-1}$. Thus, without the C-terminal domain, the overall $k_{cat,f}$ is lower, and the earlier composite $k_5$ step becomes rate-limiting in the forward direction. (The forward reaction of the truncated enzyme reduces to a rapid equilibrium condition, as suggested by the altered MgPPi inhibition data.) The step affected by the C-terminal domain must lie within the sequence $EAB \rightleftharpoons EPQ \rightleftharpoons P + EQ$. The only forward reaction step common to this sequence and the one shown earlier for the effect of the domain on the binding of monovalent oxyanions is isomerization of a ternary complex.

Activation Energies—Plots of log $V_{max}$ (APS synthesis) versus 1/T (K$^{-1}$) were linear over the range of 15–30 °C and yielded Arrhenius activation energies, $E_a$, of 17.3 and 16.9 kcal/mol for the wild type and truncated enzymes, respectively. The corresponding $\Delta H^\circ$ values (calculated as $\Delta H^\circ = E_a - RT$) were 16.7 and 16.3 kcal/mol. $\Delta G^\circ$ values calculated from absolute reaction rate theory (40) were 16.3 and 17.4 kcal/mol for the two enzyme types, respectively, at 30 °C. Thus, the entropies of activation calculated from $\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T$ were +1.3 and –3.5 entropy units/mol for the wild type and truncated enzymes, respectively. In structural terms, the more negative $\Delta S^\circ$ for the truncated enzyme could mean that the C-terminal domain assists in the orientation of the substrates at the active site, a role consistent with the comparative kinetic properties of the two forms described above.

**DISCUSSION**

*P. chrysogenum* ATP sulfurylase missing the C-terminal allosteric domain is catalytically active, but it is monomeric and much less stable than the hexameric wild type enzyme. As expected, the truncated enzyme does not display cooperativity in the presence of PAPS, at low pH, or after preincubation with an SH-reactive reagent, but in addition, truncation results in (a) a major reduction in $k_{cat}$ for the physiological reaction and marked increases in (b) the substrate Michaelis constants, (c) $k_i$ values for monovalent oxyanion inhibitors competitive with sulfate, (d) $K_i$ for MgPP$\gamma$P, and (e) $K_{i_p}$ for APS with (f) little or no change in $K_{i_a}$ and $K_{i_b}$ values. The decrease in $k_{cat}$ and the increased $K_{i_p}$ values for MgATP and sulfate result in part from (g) a large decrease in the composite $k_5$ step. These kinetic differences indicate that in addition to providing the binding site for PAPS and stabilizing the hexameric structure, the C-terminal domain also participates in perfecting the active site. This “activating” effect is focused on a step that follows the formation of the first central complex (EAB) but precedes the release of MgPP$\gamma$P. The step may be the alignment of the partially positive $\alpha$-phosphorous of MgATP with a negative oxygen of bound oxyanion. When X is sulfate (or molybdate, tungstate, etc.), catalysis then occurs. But when the first ternary complex contains thiosulfate or chloride, etc., the structural change induced by the C-terminal domain just produces a tighter dead end complex. In the absence of the C-terminal domain, the post-EAB reaction between MgATP and sulfate or molybdate still occurs, but more slowly. If the first ternary complex of the truncated enzyme contains a nonreactive monovalent oxyanion, the subsequent isomerization is diminished or may not occur at all. Standard biochemistry texts generally do not credit quaternary structure as contributing to the function or efficiency of the catalytic site (unless, of course, the site lies at a subunit interface). However, the literature does contain examples of subunit interactions that help to perfect a noninterface catalytic site (15, 41, 42).

A simple scenario for the allosteric transition of the fungal ATP sulfurylase would have the allosteric domains hold the oligomeric enzyme in a conformation where all subunit catalytic sites have the same high “proficiency” or overall catalytic competence (i.e., the R state structure). When the allosteric
inhibitor binds, stabilizing linkages are broken, and the oligomer undergoes a transition to the low proficiency T state. The model suggests that the catalytic site of truncated \textit{P. chrysogenum} ATP sulfurylase might have T state characteristics. The experimental results are consistent with this concerted transition model to the extent that the Michaelis constants of the truncated enzyme for MgATP and sulfate are increased, but the bireactant kinetics of the wild type enzyme (43) suggests that \( K_m \) of the T state is also increased, and that is not observed for the truncated enzyme.

The importance of the C-terminal domain (or part of it, at least) to structure and function is further exemplified by yeast ATP sulfurylase (12, 13). This enzyme has a hexameric structure that is very similar to that of the \textit{P. chrysogenum} enzyme. In fact, the N-terminal and catalytic domains of the two enzymes (residues 1–395) are 67\% identical in sequence and superimpose with an root mean square deviation of 0.72 Å for 363 equivalent \( \alpha \)-carbons. Yeast and \textit{P. chrysogenum} enzymes have very similar kinetic properties (14) except for their responses to PAPS (Fig. 3). At first glance, there appear to be few similarities between the C-terminal domains of the \textit{P. chrysogenum} and yeast ATP sulfurylases. The sequences do not align, and the latter is about 50 residues shorter. Nevertheless, the topology of the yeast C-terminal domain reveals that it too must have evolved from APS kinase (Fig. 5). However, the yeast enzyme is not allosterically inhibited by PAPS. This is not surprising, considering that yeast ATP sulfurylase lacks many C-terminal residues responsible for sulfonucleotide binding. For example, the mobile lid element that forms half of the binding site for (P)APS in true APS kinase (15) and in the allosteric domain of \textit{P. chrysogenum} ATP sulfurylase (9) is completely deleted in the yeast enzyme. The degenerate C-terminal domain of the yeast enzyme may be a vestigial feature of an ancestral bifunctional “PAPS synthetase,” parts of which have been retained to stabilize the hexameric structure and to hold the catalytic domain in a (perpetual) high proficiency conformation.\(^2\)

\(^2\) The classical “concerted transition” or “symmetry” model for cooperative enzymes considered only unireactant enzymes (18–20). An extension of the model to multireactant enzymes introduces additional features. For example, positive cooperativity would be observed with a bireactant enzyme even if the T and R states have identical affinities for substrates A and B (in forming the binary EA and EB complexes) as...
properties of a chimeric enzyme composed of the N-terminal and catalytic domains of the P. chrysogenum enzyme joined to the C-terminal domain of the yeast enzyme (and vice versa) would be informative.

Among ATP sulfurylases of sulfate assimilators, the enzymes from filamentous fungi and yeast may be maximally optimized for the APS synthesis direction. These hexameric enzymes have the highest APS synthesis/ATP synthesis $k_{cat}$ ratio ($-0.14$) of the several ATP sulfurylases that we have kinetically characterized so far (22, 25, 34, 38), and the $K_i$ and $K_m$ values for MgATP and SO$_4$$^2$\textsuperscript{-} are in the likely intracellular concentration range (approximately millimolar). The C-terminal domain may be the agent responsible for the extra “tailoring” of the active site. Of course, optimization must remain under the constraint of the Haldane equation, a relationship that relates the kinetic constants of the enzyme to the equilibrium constant of the reaction (see Table I). If evolutionary pressure operated to maximize the forward/reverse $k_{cat}$ ratio and, at the same time, ensure a substantial fraction of $V_{max}$ fixed at cellular levels of ATP and SO$_4$$^2$\textsuperscript{-}, then in the face of the extremely small (and unalterable) $K_m$ for the APS synthesis direction, these hexameric enzymes would be informative.

For these reasons, the R and T states of multireactant cooperative enzymes are in progress. These may reveal the structural differences in the catalytic domain that are caused by the absence of the C-terminal regulatory domain.

APPENDIX

Kinetic constants of ATP Sulfurylase

The kinetic mechanism of fungal ATP sulfurylase at pH 8 and 30 °C can be described as a random A-B, ordered P-Q sequence. The reaction scheme is shown in Scheme I. Positive and negative rate constants correspond to the “forward” and “reverse” directions, respectively.

A velocity equation that is first degree with respect to substrate concentrations has been derived assuming that $E$, $A$, and $B$ are at equilibrium with the $EA$ and $EB$ complexes (37). For this mechanism, the rate constant compositions of the limiting macrokinetic constants are as follows.

$$k_{cat} = \frac{k_{cat}\alpha}{(k_\beta + k_\delta)} \quad (Eq. 4)$$

$$k_{cat,\alpha} = \frac{(k_\beta + k_\delta)}{k_\alpha} \quad (Eq. 5)$$

$$K_{\alpha} = \frac{k_\delta}{k_\alpha} \quad (Eq. 6)$$

long as the R state has a greater degree of substrate binding synergism (21, 45). In this case, the higher affinity of the R state refers only to the formation of the ternary $EAB$ complexes. Furthermore, a Michaelis constant might be composed of more than the $k_{cat}$ and $k_{cat,\alpha}$ rate constants. For these reasons, the R and T states of multireactant cooperative enzymes are best referred to in terms of their overall catalytic competencies (or effectiveness or efficiencies) rather than in the terms of their "affinities."
**Kinetic and Stability Properties of *Penicillium chrysogenum* ATP Sulfurylase Missing the C-terminal Regulatory Domain**

Eissa Hanna, Kit Fai Ng, Ian J. MacRae, Christopher J. Bley, Andrew J. Fisher and Irwin H. Segel

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