Induction of Positive Cooperativity by Amino Acid Replacements within the C-terminal Domain of \textit{Penicillium chrysogenum} ATP Sulfurylase*

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ATP sulfurylase from \textit{Penicillium chrysogenum} is an allosteric enzyme in which Cys-509 is critical for maintaining the R state. Cys-509 is located in a C-terminal domain that is 42\% identical to the conserved core of adenosine 5'-phosphosulfate (adenylylsulfate) (APS) kinase. This domain is believed to provide the binding site for the allosteric effector, 3'-phosphoadenosine 5'-phosphosulfate (PAPS). Replacement of Cys-509 with either Tyr or Ser destabilizes the R state, resulting in an enzyme that is intrinsically cooperative at pH 8 in the absence of PAPS. The kinetics of C509Y resemble those of the wild type enzyme in which Cys-509 has been covalently modified. The kinetics of C509S resemble those of the wild type enzyme in the presence of PAPS. It is likely that the negative charge on the Cys-509 side chain helps to stabilize the R state. Treatment of the enzyme with a low level of trypsin results in cleavage at Lys-527, a residue that lies in a region analogous to a PAPS motif-containing mobile loop of true APS kinase. Both mutant enzymes were cleaved more rapidly than the wild type enzyme, suggesting that movement of the mobile loop occurs during the R to T transition.

ATP sulfurylase (MgATP:SO\textsubscript{4}\textsuperscript{2-} adenyllytransferase, EC 2.7.7.4) catalyzes the first intracellular reaction in the incorporation of inorganic sulfate into organic molecules by sulfate assimilating organisms:

\[
\text{MgATP} + \text{SO}_4^{2-} \rightleftharpoons \text{MgPP} + \text{APS}
\]

APS\textsuperscript{3-} is then phosphorylated to PAPS in a reaction catalyzed by the second sulfate-activating enzyme, APS kinase, (MgATP: adenosine 5'-phosphosulfate 3' phosphotransferase EC 2.7.1.25):

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

ATP sulfurylase from the filamentous fungus \textit{Penicillium chrysogenum} is an oligomer composed of six identical 64-kDa subunits (573 residues). Each subunit possesses three free SH (cysteiny1) groups,\textsuperscript{2} of which only one (designated SH-1) can be modified by sulfhydryl-reactive reagents such as DTNB and NEM under non-denaturing conditions (1). Complete modification of SH-1 (six per hexamer) changes the initial velocity kinetics at pH 8 from normal-hyperbolic (Hill coefficient, \(n_H = 1\)) to sigmoidal (\(n_H \approx 2\)) with a concomitant increase in the \([S]_{0.5}\) values for MgATP and SO\textsubscript{4}\textsuperscript{2-} (or MoO\textsubscript{4}\textsuperscript{2-}); \(V_{\text{max app}}\) at a fixed subsaturating cosubstrate level is reduced (2). A number of experimental approaches, including protection against chemical inactivation by reversibly bound ligands (2), direct binding measurements (3), and single turnover isotope trapping (3), established that the sigmoidal curves reflected true cooperative binding as opposed to a kinetically based phenomenon.

The dramatic effect of \textit{in vitro} modification of SH-1 suggested several possible scenarios, including that modification induces a conformational state in the enzyme that is normally induced \textit{in vivo} by a reversibly bound allosteric effector. The effector was subsequently shown to be PAPS (4). Further experiments established that the enzyme from several other fungi behaved identically to the \textit{P. chrysogenum} enzyme, whereas ATP sulfurylases from rat liver (5), spinach leaf (6), cabbage leaf (7), yeast (4), and the \textit{Riftia} bacterial symbiont (8) did not respond in the same way to Cys modification or to PAPS. The cumulative results indicated that \textit{(a)} fungal ATP sulfurylase possesses an allosteric PAPS binding site that is not present in the enzyme from other sources and \textit{(b)} SH-1 is either in the region of, or in communication with, the PAPS binding site. Fungal sulfurylase was subsequently shown to possess a C-terminal region (approximately residues 396–539) that is 42\% identical to the conserved core of APS kinase (9–11), a protein with a high affinity for PAPS. SH-1 is Cys-509, which is located in the APS kinase-like C-terminal domain, a few residues upstream from a putative PAPS motif (12). It is likely that residues 396–540 of \textit{P. chrysogenum} ATP sulfurylase evolved from true APS kinase and that this region provides the allosteric binding site for PAPS. In effect, the C-terminal region of fungal ATP free enzyme; R and T states, “relaxed” and “taut” structural states of allosteric enzymes.

\textsuperscript{2} Among fungal ATP sulfurylases that have been examined so far, two Cys residues (Cys-42 and Cys-509) are conserved. The third one in \textit{P. chrysogenum} (Cys-68) replaces a Val that is present at that position in other fungal ATP sulfurylases.
sulfurylase is a regulatory subunit that happens to be co- 
valently linked to the catalytic subunit.3 Our preliminary hy- 
thesis (in terms of the concerted transition model) was that 
covalent modification of Cys-509 promotes the same R to T 
allostERIC transition (13, 14) as does PAPS binding. 

The inhibition of P. chrysogenum ATP sulfurylase by PAPS 
may be the way that fungi prevent PAPS accumulation to toxic 
levels. Another consideration is that in fungi, PAPS is a major 
branch point metabolite of sulfate assimilation. One branch 
leads to cysteine and other reduced sulfur compounds; the 
other branch to choline-O-sulfate, a sulfur storage compound 
and/or osmoprotectant (15–18). Thus the inhibition may be 
part of a more extensive sequential feedback process. In 
contrast, yeasts and most bacteria do not form large quantities 
of sulfate esters, whereas plants (and some bacteria) preferentially 
use APS (rather than PAPS) as the substrate for the 
reductive assimilation of sulfate. In other words, PAPS is not 
at a branch point in these other organisms.

The objective of the present study was to establish the role of 
Cys-509 in stabilizing the R state. To this end, we investigated 
the kinetic consequences of replacing Cys-509 with either ty- 
rosine or serine.

MATERIALS AND METHODS

Introducing Mutations—Mutations in codon 509 were made by PCR 
amplification of the C-terminal 221 base pairs of the fungal ATP sul- 
furylase gene (codons 506–573). This sequence begins with an inde- 
finite XhoI site 3 base pairs upstream from codon 509 and ends after the 
stop codon with an engineered XbaI site. Each PCR used a cloned cDNA 
copy of the native gene as the template, the C-terminal coding primer 
PcATS308 (5'-GGTTGCTATCTTATGGCCAGTGGGAAAACCC-3'), 
and an upstream primer containing the XhoI site and the desired muta- 
tions. Primers bear with their respective produced mutations 
were as follows: PcATS313 (C059S), 5'-TCCCTCGAGAAGCTCTGAGC- 
AGTCCG-3' and PcATS317 (C509Y), 5'-GCCCCTCAGACAAGCACAGA- 
GTCGG-3'. All PCRs were carried out using the DNA polymerase Pfu 
(Stratagene). The resulting 221-base pair DNAs were subcloned as 
XhoI-XbaI fragments into a pBluescript KS(+) plasmid and introduced into 
Escherichia coli strain BL21 (DE3) for protein expression.

Protein Expression and Purification—About 0.2 ml of an 8-h culture 
was used to inoculate two 3-liter Fernbach flasks each containing 1000 
ml of LB ampicillin medium. The cultures were grown aerobically at 
37 °C for 8–10 h and then transferred to 15 °C. Upon transfer to 15 °C, 
1 g of o-lactose was added per liter of culture to induce protein 
expression. After 8–10 h at 15 °C, the cells were harvested by centrifugation 
at 12,000 × g for 10 min. Approximately 4–8 ml of packed cells was 
obtained. The cells were then resuspended in about 50 ml of chilled 
40 mM Tris-Cl, pH 8.0, and lysed in a single pass through a Watts Fluidar 
Microfluidizer (model B12–04DJC M3). All subsequent steps were 
carried out at 4 °C. Cell debris and unbroken cells were removed by 
centrifuging at 16,000 × g for 10 min. The supernatant fluid was 
applied to a blue dextran (19) column (2.5 × 10 cm) that had been 
equilibrated with 40 mM Tris-Cl, pH 8.0. The column was then washed 
with the same buffer at 6 ml/min until the effluent had an A280 of no 
0.005 or less. Protein was eluted with a linear gradient of NaCl (0–0.7 
m) in 40 mM Tris-Cl, pH 8.0 (total volume 500 ml) at a flow rate of 2 ml 
per min. 7-m1 fractions were collected, and their A280 nm and ATP 
sulfurylase activity were measured. Fractions containing enzyme activ- 
ity (coincident with the major protein peak) were pooled (total volume 
approximately 85 ml), dialyzed against 40 mM Tris-Cl, pH 8.0, and then 
and applied to a DEAE-cellulose column (2.5 × 10 cm) equilibrated in 
the same buffer. After a brief wash, protein was eluted at 1 ml per min with 
a linear gradient of NaCl (0–0.4 m) in 40 mM Tris-Cl, pH 8.0 (total 
volume, 400 ml). Seven fractions containing ATP sulfurylase activity 
totaling 49 ml were pooled, divided into 1-ml aliquots, and stored 
frozen. A typical preparation yielded about 25 mg of pure enzyme. The 
A280 nm/A405 nm ratio of the enzymes ranged from 1.91 (for C509Y) to 
2.01 (for C509S). SDS gel electrophoresis indicated that all the enzymes 
were at least 95% pure. The absence of Cys-509 in the mutant enzymes 
was confirmed by demonstrating their lack of reactivity with DTNB 
in the absence of SDS (1). 

Enzyme Assays—ATP sulfurylase activity was characterized by the 
continuous, coupled spectrophotometric molybdenum assay (22) in the 
presence of NADH, P-enolpyruvate, KCl, excess adenylate kinase, in- 
organic pyrophosphatase, sulfate-free pyruvate kinase + lactate dehy- 
drogenase, and approximately 0.5 μg (0.02 unit) of pure P. chrysogenum 
APS kinase (10, 22, 23). The stoichiometry of the assay is 2 mol of 
NADH oxidized per mol of AMP formed. In addition to providing good 
sensitivity, this assay has the advantage in that both primary sub- 
strates, MgATP and MoO42-, are continuously regenerated. The APS 
kinase serves to remove traces of APS formed from contaminating 
inorganic sulfate during the preincubation period (20). (APS is a potent 
product inhibitor of the enzyme, whereas the small increment of PAPS 
formed is innocuous.) Unless indicated otherwise, all assays were con- 
ducted at 30 °C, in 50 mM Tris-Cl, pH 8.0. The total MgCl2 present 
was always 5 mM greater than that of the total ATP. The specific activities 
of the wild type, C509S, and C509Y forms of the enzyme freshly purified 
from E. coli expression system and assayed at 5 mM total ATP, 10 
mM MgCl2 (as MgCl2), and 10 mM MoO42- were, in order, 20, 17, and 
14.5 units × mg of protein-1. 1 unit is the amount of enzyme that 
catalyzes the formation of 1 μmol of primary product in 1 min.

Data Analysis—For each experimental velocity curve, the Vmax value 
and the Hill coefficient, nH, were determined by fitting the plotted v 
versus [substrate] data to the Hill equation:

\[
\frac{v}{V_{\text{max}}} = \frac{[S]^n}{K + [S]^n} \quad \text{(Eq. 1)}
\]

Hill coefficients were also determined as the slope of the Hill plot,

\[
\log \frac{v}{V_{\text{max}}} = n_H \log [S] - \log K_H \quad \text{(Eq. 2)}
\]

in the region corresponding to 50% saturation (i.e. where log [v/V_{\text{max}}] = 0) or over the range corresponding to 10–90% saturation (14).

RESULTS

Kinetic Properties of Cys-509 → Tyr—Fig. 1 shows the veloc- 
ity curves of the C509Y mutant enzyme under standard assay 
conditions. The most striking feature of the curves is that the cur- 
vatures in the absence of PAPS. In fact, the increase in 
with increasing concentrations of the fixed cosubstrate is 
the same trend displayed by the wild type enzyme after cova-
lent modification of Cys-509 (data not shown). Up to this point, the results suggested that cooperative behavior is induced by either (a) increasing the bulk of the side chain at position 509 or (b) eliminating the negative charge (R-S⁻) at this position. It was thought that replacing Cys-509 with the slightly smaller and uncharged Ser might help to distinguish between these two possibilities.

**Kinetic Properties of Cys-509**

The native enzyme modified with NEM at Cys-509 yielded the following data: The $n_H$ of the $v$ versus $[\text{MgATP}]$ plots increased from 1.2 at 0.2 mM $\text{MoO}_4^{2-}$ to 2.0 at 5 mM $\text{MoO}_4^{2-}$. The $n_H$ of the $v$ versus $[\text{MoO}_4^{2-}]$ plots increased from 1.8 at 0.3 mM MgATP to 2.1 at 5 mM MgATP.

**Fig. 1. Velocity curves of C509Y.** A, $v$ versus $[\text{MgATP}]$ at pH 8.0 and the indicated fixed concentrations of molybdate. B, $v$ versus $[\text{MoO}_4^{2-}]$ at pH 8.0 and the indicated fixed concentrations of MgATP.

**Fig. 2. Velocity curves of C509S.** A, $v$ versus $[\text{MgATP}]$ at pH 8.0 and the indicated fixed concentrations of molybdate. Inset, velocity curve at 7.5 mM $\text{MoO}_4^{2-}$. B, $v$ versus $[\text{MoO}_4^{2-}]$ at pH 8.0 and the indicated fixed concentrations of MgATP. Inset, velocity curve at 5 mM MgATP over a narrower $\text{MoO}_4^{2-}$ concentration range. Curve fits of the 5 mM MgATP data to the Hill equation returned $n_H$ values of 1.04 to 1.05 (depending on the range covered). However, curve fits to the Henri-Michaelis-Menten equation (which fixes $n_H$ at 1.00) were, for all practical purposes, equally good ($R^2$ in both cases was $>0.999$).

and uncharged Ser might help to distinguish between these two possibilities.
ity curves of the C509S enzyme at several different fixed concentrations of cosubstrate. Despite the size similarity of Ser and Cys, the plots are again sigmoidal, although, compared with C509Y, C509S has a lower $S_{0.5}$ for either substrate at any given concentration of cosubstrate. Also, unlike the curves shown in Fig. 1, the $n_H$ values of the $v$ versus $[MgATP]$ plots for C509S do not change significantly with increasing [MoO$_4^{2-}$]. At subsaturating MgATP, the $v$ versus $[MoO_4^{2-}]$ curves are also sigmoidal, but $n_H$ approaches unity as the concentration of MgATP approaches saturation. This trend is consistent with the preferential binding of MgATP to free E of the R state. That is, as the fixed [MgATP] approaches saturation, the enzyme is driven far toward the T state than $v$ versus $[S]$, which is the same as that of the noncooperative wild type enzyme. In terms of Equation 9 (Appendix), $L_{app}$ for C509S at saturating MgATP (equivalent to $L_c$) must be very small, implying that $c$ is less than unity. The sigmoidicity of the $v$ versus $MoO_4^{2-}$ plot at subsaturating MgATP can be attributed, at least in part, to the synergism between MgATP and MoO$_4^{2-}$. That is, even if $K_{bR} = K_{bRT} (e = 1)$, and $K_{bRT} = K_{bRT} (f = 1)$, the $v$ versus $[B]$ plots can be sigmoidal at subsaturating $[A]$ if (a) substrate $A$ binds preferentially to the R state ($c < 1$), and (b) the substrates bind to the R state synergistically ($f < 1$). The last condition seems highly likely given that the R state should closely resemble the noncooperative wild type enzyme where the $K_c$ value for each substrate is smaller than the corresponding $K_v$ value.

The bieactant kinetics of C509S are similar to those of the wild type enzyme in the presence of PAPS. Comparing the above results with those of the C509Y enzyme leads to the conclusions that either (a) substituting a Tyr residue for Cys-509 drives the enzyme much further toward the T state than does substituting a Ser at this position or (b) the T state induced by substituting Tyr at position 509 is structurally different from that induced by substituting Ser (see “Discussion”). In either case, the results show that cooperative behavior is a not simply a result of increasing the bulk of the residue at position 509. Either the negative charge on the side chain of Cys-509 plays a critical role in stabilizing the R state, or the side chain size is extremely important and any change will favor a shift to the T state.

**Effect of a Competitive Inhibitor—**Activation by a competitive inhibitor at low competitive substrate concentrations is a hallmark of true cooperative binding. As shown in Fig. 3, inorganic thiosulfate, an inhibitor competitive with $SO_4^{2-}$ or MoO$_4^{2-}$ (24), does exactly that. Activation by $S_2O_3^{2-}$ is also seen with the wild type enzyme after chemical modification of Cys-509 (2), or in the presence of PAPS (4, 20). Note that the experimental level of the noncompetitive cosubstrate (MgATP) influences the effect of the competitive inhibitor. That is, the activation is eliminated by an MgATP concentration that is too low in the case of C509Y, or too high in the case of C509S. These opposite effects are consistent with the different effects of MgATP binding on the cooperativity of the two mutant enzymes as illustrated in Figs. 1b and 2b.

The wild type enzyme yielded the following data at 50 $\mu$M PAPS: The $n_H$ of the $v$ versus $[MgATP]$ plots varied from approximately 1.5 at 0.1 mM MoO$_4^{2-}$ to 1.7 at 1 mM MoO$_4^{2-}$. The $n_H$ of the $v$ versus $[MoO_4^{2-}]$ plots decreased from approximately 2 to 0.05 mM MgATP to approximately 1 at 2.5 mM MgATP.

Exactly how “low” the competitive substrate must be to demonstrate activation is best established by trial and error. For a simple unreactant system where $S$ and I bind exclusively to the R state, the peak velocity occurs at $\theta = \sqrt{L_{bR} - 1} - 1 = 1$, where $\theta = [I]/K_s$ and $\alpha = [S]/K_v$. Thus as the fixed $[S]$ is increased, the peak moves closer to the vertical axis and eventually disappears.

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**Table I**

| pH   | $n_H$ | $[MoO_4^{2-}]_{1/2}$ | $V_{max, app}$ | $\mu$mol $\times$ min$^{-1}$ $\times$ mg of protein$^{-1}$ |
|------|-------|-----------------------|----------------|---------------------------------------------------------|
| 8.0  | 1.06  | 0.32                  | 17.2           |
| 7.5  | 1.16  | 0.42                  | 16.3           |
| 7.0  | 1.66  | 0.48                  | 14.2           |
| 6.5  | 2.30  | 0.72                  | 12.8           |

**Kinetics at Lower pH—**The side chain of a Ser residue is not much smaller than that of a Cys residue, but unlike Ser, a substantial fraction of the Cys side chain may be ionized at the standard assay pH of 8.0. The observation that C509S is intrinsically cooperative raised the possibility that the charge on residue 509 plays a major role in stabilizing the R state. If the side chain of Cys-509 behaves normally (i.e., has a $pK_a$ of 8.0–8.5), decreasing the assay pH from 8.0 to (e.g.) 6.5 would decrease the fraction of the residue in the Cys-$S^-$ form significantly. It was of interest then to determine whether protonating the Cys anion of the wild type enzyme had the same effect as substituting Ser for Cys. As shown in Table I, decreasing the pH did indeed induce sigmoidal $v$ versus $[S]$. However, the extent of induction was much less than that of the C509Y enzyme. For example, at pH 7.0, the $n_H$ value for the C509Y enzyme was 1.06, while for C509S, it was only 1.16. These results suggest that the influence of the Cys-509 side chain on enzyme stability is minimal at pH 8.0, but is significant at pH 7.0. Additionally, the Hill coefficient for C509S at pH 7.0 was 1.16, which is close to the value expected for a noncooperative enzyme. At pH 8.0, the Hill coefficient was 1.06, which is close to the value expected for a cooperative enzyme.

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![Graphs showing enzyme activity at different pH levels](image-url)
The experiments described in Table I were conducted in MES-Tris buffers in which the MES concentration increased as the pH was decreased. However, MES per se was not responsible for the sigmoidicity as evidenced by the hyperbolic velocity curves obtained in 0.05M MES (plus Tris to pH 8).

Effect of PAPS on C509S—It was of interest to determine whether PAPS had an additional effect on a mutant enzyme, or whether the mutation transformed the enzyme completely to the T state. As shown in Fig. 4, the $n_H$ value of C509S increased further as the concentration of PAPS was increased. At 240 μM [PAPS], the $n_H$ of the $v$ versus [MgATP] plot was nearly 3. Thus the Cys to Ser mutation promoted only a partial shift toward the T state allowing the R to T equilibrium to be driven further toward the T state or back toward the R state by the appropriate ligand. In this respect, C509S resembles a typical allosteric enzyme. The apparent $n_H$ limit of 3 (instead of 6) is very likely a consequence of the nonexclusive binding of PAPS and/or substrates. However, the possibility that the enzyme behaves in an alternating “half-of-the-sites” manner cannot be immediately discarded. The effect of PAPS on $V_{max, app}$ indicates that either (a) the catalytic activity of the T state is much less than that of the R state, or (b) substrate binding to the T state is not highly synergistic, or (c) both conditions apply. In contrast to the results shown in Fig. 4, PAPS decreased the sigmoidicity of the $v$ versus [MgATP] plot of C509Y. At 1 mM MoO$_4^{2-}$ in the absence of PAPS, $n_H$ and $V_{max, app}$ were, respectively, 2.3 and 12.2 units $\times$ mg of protein$^{-1}$. At 240 μM PAPS, $n_H$ was 2.0; $V_{max, app}$ decreased to 6.8 units $\times$ mg of protein$^{-1}$ (data not shown).

Susceptibility of the C-terminal Domain to Proteolysis—As shown in Fig. 5, treatment of wild type $P.\text{chrysogenum}$ ATP sulfurylase with a low concentration of trypsin results in an initial rapid cleavage producing a well-defined product. Sequence analysis of the products revealed that the primary site of cleavage was at Lys-527, a residue that lies in a region analogous to the PAPS motif-containing mobile loop of true APS kinase and close to the analogous “quick trypsin” site of that enzyme (which is Arg-158) (11). In some incubations, cleavage at a second “quick trypsin” site of ATP sulfurylase (Arg-488) could be detected before the pattern was obscured by further proteolysis. MgATP, APS, or PAPS protected the wild type and C509S against proteolysis. C509Y was not protected. Further proteolysis. MgATP, APS, or PAPS protected the wild type and C509S against proteolysis. C509Y was not protected. The pattern for the wild type enzyme in the presence of PAPS is shown in the second row of Fig. 5. Both mutant enzymes were cleaved much more rapidly than the wild type enzyme, suggesting that the mobile loop/PAPS motif region is more accessible in the T state than in the R state. (The pattern for C509S is shown in the third row of Fig. 5.) Considering the sequence homology of the two enzymes and the similar locations of the primary “quick trypsin” sites (Fig. 6), it is likely that true APS kinase and the C-terminal domain of ATP sulfurylase have similar structures.
DISCUSSION

Data obtained in the present study indicate that Cys-509 participates in stabilizing fungal ATP sulfurylase in an R state, which binds both substrates hyperbolically with high affinity. The requirement for Cys at position 509 is quite strict. Replacing Cys-509 with tyrosine promotes the transition to a low affinity T state. As a result, the \( v \) versus [MgATP] and \( v \) versus [MoO\(_4\)]\(^2\) curves are sigmoidal (in the absence of PAPS) with Hill coefficients, \( n_H \), that increase as the concentration of the fixed substrate is increased. In this respect, C509Y behaves like the wild type enzyme covalently modified at Cys-509 by fixed substrate is increased. In this respect, C509Y behaves like the wild type enzyme covalently modified at Cys-509 by DTNB, NEM, or tetrathionate. Substituting serine at position 509 also destabilizes the R state and again the result is sigmoidal velocity curves. The \( v \) versus [MgATP] curve remains sigmoidal at saturating MoO\(_4\)]\(^2\), but the \( v \) versus [MoO\(_4\)]\(^2\) curve becomes hyperbolic at saturating MgATP. These kinetics indicate that MgATP has a higher affinity for the free E of the R state compared with its affinity for free E of the T state, but that cosubstrate MoO\(_4\)]\(^2\) binds more or less equally well to free E of both states. Stated alternatively, MgATP alone can trigger the T to R transition of C509S. The kinetic effects of substituting Ser at position 509 are the same as those promoted by the reversible binding of PAPS to the wild type enzyme.

At first glance, there appears to be two different classes of kinetic response to alterations at position 509. The simplest explanation for the different kinetics is that there is a single T state, but different alterations in the region of Cys-509 cause a different extent of R to T transition, i.e. result in different base level values of the allosteric constant, \( L \) (13). The consequence of the difference is best appreciated by examining a plot of \( n_H \) versus log \( L \). If the T state has catalytic activity (even very low compared with the R state), the plot is bell-shaped with limits of 1.0 (14, 27). The effect of increasing the concentration of a ligand on \( n_H \) depends on which side of the maximum the enzyme is poised in the absence of ligands, i.e. whether the base level \( L \) is larger or smaller than the \( L \) at the maximum \( n_H \). Thus a decrease in the apparent \( L \) value (as would occur when the fixed concentration of a cooperatively bound cosubstrate is increased) could result in an increase or a decrease in \( n_H \). Whatever the effect of the fixed substrate concentration on \( n_H \), an increase in the apparent \( L \) (as would occur when the concentration of the allosteric inhibitor is increased) will have the opposite effect. If this explanation is applicable, the different \( [S]_{0.5} \) values of the two mutant enzymes and the effects of changing [MgATP] or [PAPS] on \( n_H \) mean that covalently modifying or protonating the wild type enzyme, or replacing Cys-509 with Tyr drives the enzyme further toward the T state than does PAPS binding or replacement of Cys-509 with Ser.

Another possible cause of the two classes of kinetics is that there are two types of T states. One type is produced by substituting Tyr for Cys at position 509, or by covalently modifying Cys-509 of the wild type enzyme, or by decreasing the pH below 8. The second type is formed when Cys-509 is replaced by Ser or when the wild type enzyme binds PAPS. In this scenario, the R to T transition of C509Y would be driven mainly by the formation of the R state ternary E-MgATP-MoO\(_4\)]\(^2\)\(^-\) complex.

Compared with the wild type enzyme, the mutant enzymes have lower specific activities at saturating MgATP and MoO\(_4\)]\(^2\)\(^-\) (wild type > C509S > C509Y). If the \( V_{max} \) values of the mutant enzymes are the same as that of the wild type enzyme, then their lower specific activities can be attributed to different base level \( L \) values, nonexclusive substrate binding, and a low activity T state (see “Appendix,” Equation 12. For example, the molybdobis (V)\(_{max} \) of C509S (17 units \( \times \) mg of protein\(^{-1} \)) is about 85% that of the wild type enzyme, suggesting that about 15% of the enzyme remains trapped in the very low activity T state at saturating substrate levels.

The dramatic change in kinetic properties resulting from the substitution of Tyr or Ser for Cys-509 confirms the key role of this position in holding the enzyme in the R state. As shown in Fig. 6, the residue analogous to Cys-509 in true APS kinase is Ala-145, which is located just before a mobile loop (residues 149–169) containing a putative PAPS motif (12). This loop is believed to serve as a hinged element (“ATP lid”) that immobilizes and protects bound MgATP in APS kinase (11). Ala-145 (and by inference, Cys-509) is located within a short helix at the N-terminal end of the loop. Although the C-terminal domain of ATP sulfurylase probably does not bind MgATP (because of alterations to the P-loop; see Ref. 10), a similar motion of the analogous mobile element may play a role in the R to T transition—a suggestion consistent with the observations that (a) the primary “quick trypsin” site resides within the PAPS motif of the mobile loop and (b) that site is more accessible in the mutant enzymes (which exist primarily in the T state) than in the wild type enzyme (which exists almost entirely in the R state). If a movement of the loop does occur as part of the allosteric transition, one can understand why covariant modifi-

\(^7\) A number of years ago, Pettigrew and Frieden (30) warned that “the assumption that effects of the second substrate upon kinetic behavior may be ignored as long as it is at a saturating concentration may be invalid and lead to incorrect predictions. . . .” The experimental effects of the nonvaried substrate on cooperativity presented in this present report (Figs. 1 and 2) confirm that warning.
cation or amino acid substitution within the small helix (hinge?) might alter the allosteric equilibrium. The facile dissociation and reassociation of subunits is another physical characteristic of APS kinase (28) that may have been recruited by ATP sulfurylase as part of the allosteric transition. Indeed, preliminary x-ray diffraction studies indicate that *P. chrysogenum* ATP sulfurylase has a dimer-of-triads structure, which is partially stabilized by interactions of C-terminal domains across the triad interface.

**APPENDIX**

**Kinetic Behavior of a Bireactant Cooperative Enzyme**

The principles of the concerted transition (symmetry) model for cooperative enzymes (13, 27) can be extended to multireactant enzymes provided that rapid equilibrium conditions prevail (or are assumed) for the substrate binding steps and the allosteric transition (29). Compared with unireactant systems, the requirement that both substrates bind to the enzyme before any catalytic activity occurs adds another layer of complexity. For example, one or both of the substrates might bind cooperatively to the free enzyme, but neither substrate might bind cooperatively to the binary enzyme-cosubstrate complex. Conversely, only one or both of the substrates might bind cooperatively to the enzyme-cosubstrate complex, but neither might bind cooperatively to the free enzyme. Also, the binding of one substrate at the catalytic site may promote or may hinder the binding of the other substrate. This heterotrophic interaction can also affect the properties of the velocity curves. Because of these possibilities, the Hill coefficient for the varied substrate might increase, decrease, or remain the same as the concentration of the nonvaried substrate is increased.

The velocity equation for bireactant ATP sulfurylase in the presence of substrates A (MgATP) and B (MoO₄²⁻), which add in a rapid equilibrium random fashion, is shown below. The equation takes into account that X (PAPS), the allosteric effector, binds to the catalytic site as an inhibitor competitive with both MgATP and MoO₄²⁻, as well as to the allosteric site (20),

\[
v = \frac{\text{num}_1 + \text{num}_2}{\text{denom}_1 + \text{denom}_2} \quad \text{(Eq. 3)}
\]

where

\[
\text{num}_1 = V_{\text{max}R} \left( 1 + \frac{[X]}{K_{XR}} \right)^n \left( \frac{[A][B]}{K_{aB}K_{bR}} \right) \left( 1 + \frac{[A]}{K_{aA}} + \frac{[B]}{K_{aB}} + \frac{[A][B]}{K_{aA}K_{bB}} + \frac{[X]}{K_{XR}} \right)^{-1} \quad \text{(Eq. 4)}
\]

\[
\text{num}_2 = V_{\text{max}T} \left( 1 + \frac{[X]}{K_{XT}} \right)^n \left( \frac{[A][B]}{K_{aB}K_{bR}} \right) \left( 1 + \frac{[A]}{K_{aA}} + \frac{[B]}{K_{aB}} + \frac{[A][B]}{K_{aA}K_{bB}} + \frac{[X]}{K_{XR}} \right)^{-1} \quad \text{(Eq. 5)}
\]

\[
\text{denom}_1 = L \left( 1 + \frac{[X]}{K_{XR}} \right)^n \left( 1 + \frac{[A]}{K_{aA}} + \frac{[B]}{K_{aB}} + \frac{[A][B]}{K_{aA}K_{bB}} + \frac{[X]}{K_{XR}} \right)^n \quad \text{(Eq. 6)}
\]

\[
\text{denom}_2 = L \left( 1 + \frac{[X]}{K_{XR}} \right)^n \left( 1 + \frac{[A]}{K_{aA}} + \frac{[B]}{K_{aB}} + \frac{[A][B]}{K_{aA}K_{bB}} + \frac{[X]}{K_{XR}} \right)^n \quad \text{(Eq. 7)}
\]

\[K_{aA} \text{ and } K_{bB} \text{ are, respectively, the } A \text{ and } B \text{ dissociation constants of the R state EA and EB complexes. } K_{bR} \text{ is the B dissociation constant from the R state EAB complex. } K_{ab} \text{ is the PAPS dissociation constant of the R state catalytic site. } K_{ma} \text{ does not appear in the equation, but for each state, } K_{ma} \text{ equals } K_{aB}K_{bR}. \]

\[K_{XR}, K_{XT}, K_{aB}, K_{bB}, \text{ and } K_{XR} \text{ are the corresponding T state catalytic site constants. } K_{XR} \text{ and } K_{XT} \text{ are the PAPS dissociation constants of the T state and R state allosteric sites, respectively. Exponent } n \text{ is the number of subunits in the oligomer, each one bearing a catalytic and an allosteric site. (For ATP sulfurylase, } n = 6. ) L \text{ is the allosteric constant, i.e. the } [T]_0/[R]_0 \text{ ratio in the absence of ligands. } V_{\text{max}R} \text{ and } V_{\text{max}T} \text{ are the maximal velocities of the R and T states, respectively } (V_{\text{max}R} = n_{\text{kcat}R} \text{ and } V_{\text{max}T} = n_{\text{kcat}T}).\]

The \text{num}_1 term accounts for product formation by all T state complexes containing bound A and B. Similarly, \text{num}_2 accounts for product formation by R state complexes containing both A and B. The denom_1 term represents the concentrations of all T state species relative to \([R]_0\). The denom_2 term represents the concentrations of all R state species relative to \([R]_0\).

If ligand concentrations and the catalytic rate constants are normalized to their respective R state constants (a common practice in displaying equations for this model (13, 30), the velocity can be written as:

\[
v = \frac{gLC\cos \beta \left( 1 + ca + eB + \frac{\cos \beta}{h} + q\delta \right)^{-1} + \frac{abB}{f} \left( 1 + \alpha + \beta + \frac{abB}{f} + \delta \right)^{-1}}{L(1 + ca + eB + \frac{\cos \beta}{h} + q\delta)^n + (1 + \gamma)^n \left( 1 + ca + eB + \frac{\cos \beta}{h} + q\delta \right)^n \left( 1 + \alpha + \beta + \frac{abB}{f} + \delta \right)^n} \quad \text{(Eq. 8)}
\]

where

\[
a = \frac{[A]}{K_{aA}}, \quad \beta = \frac{[B]}{K_{aB}}, \quad \gamma = \frac{[X]}{K_{XR}}, \quad \delta = \frac{[A][B]}{K_{aA}K_{bB}}, \quad e = \frac{[X]}{K_{XR}}, \quad \delta = \frac{[A][B]}{K_{aA}K_{bB}};
\]

\[
f = \frac{K_{aB}K_{bR}}{K_{aB}K_{bR}}, \quad h = \frac{K_{aA}K_{bB}}{K_{aA}K_{bB}}, \quad m = \frac{K_{aA}K_{bB}}{K_{aA}K_{bB}}, \quad q = \frac{K_{aA}K_{bB}}{K_{aA}K_{bB}}, \quad g = \frac{V_{\text{max}T}}{V_{\text{max}R}}.
\]

The terms \(c, e, \) and \(m, \) are, in order, the nonexclusive binding coefficients of substrates A and B and the allosteric inhibitor, X. The interaction factor \(f\) describes the effect of the binding of one substrate on the dissociation constant of the other substrate at the catalytic R site. Thus, if \(f < 1, \) the substrates bind synergistically. The factor \(h\) is the corresponding substrate interaction factor of the T state. Other coefficients that are sometimes useful for simplifying equations or describing kinetic properties are:

\[
j = \frac{ef}{h} = \frac{K_{aB}K_{bR}}{K_{aA}K_{bB}} \quad \text{and} \quad p = \frac{K_{aB}K_{bR}}{K_{aA}K_{bB}}
\]

When substrate A is saturating, the velocity at any \([B]\) is given by:

\[
v = \frac{gV_{\text{max}L}c^e \beta h \left( 1 + \frac{[B]}{K_{XR}} \right)^{-1} + V_{\text{max}T} \frac{[B]}{K_{XR}} \left( 1 + \frac{[B]}{K_{XR}} \right)^{-1}}{L'c^e \left( 1 + \frac{[B]}{K_{XR}} \right)^n + \left( 1 + \frac{[B]}{K_{XR}} \right)^n} \quad \text{(Eq. 9)}
\]

or

\[
v = \frac{gV_{\text{max}L}c^e \beta h \left( 1 + \frac{e\beta}{h} \right)^{-1} + V_{\text{max}T} \frac{h}{B} \left( 1 + \frac{[B]}{K_{XR}} \right)^{-1}}{L'c^e \left( 1 + \frac{e\beta}{h} \right)^n + \left( 1 + \frac{[B]}{K_{XR}} \right)^n} \quad \text{(Eq. 10)}
\]

where \(L' \) is the apparent allosteric constant at the fixed \([X]\) in the absence of other ligands:

\[
L' = \left( \frac{1 + [X]}{K_{XR}} \right)^n \left( 1 + \frac{[X]}{K_{XR}} \right)^{n-1} \quad \text{(1 + \gamma)^n} \quad \text{(Eq. 11)}
\]
is less catalytically active than the R state (i.e. $g < 1$), $v_{\text{limit}}$ can be $< V_{\text{maxR}}$.

REFERENCES

1. Renosto, F., Schultz, T., Re, E., Mazer, J., Chandler, C. J., Barron, A., and Segel, I. H. (1985) *J. Bacteriol.* **164**, 674–683

2. Renosto, F., Martin, R. L., and Segel, I. H. (1987) *J. Biol. Chem.* **262**, 16279–16288

3. Martin, R. L., Daley, L. A., Lovric, Z., Wailes, M. L., Renosto, F., and Segel, I. H. (1989) *J. Biol. Chem.* **264**, 11768–11775

4. Renosto, F., Martin, R. L., Wailes, L. M., Daley, L. A., and Segel, I. H. (1990) *J. Biol. Chem.* **265**, 10300–10308

5. Yu, M., Martin, R. L., Jain, S., Chen, L. J., and Segel, I. H. (1989) *Arch. Biochem. Biophys.* **269**, 156–174

6. Renosto, F., Patel, H. C., Martin, R. L., Thomassian, C., Zimmerman, G., and Segel, I. H. (1993) *Arch. Biochem. Biophys.* **307**, 272–285

7. Ooslund, T., Chandler, C., and Segel, I. H. (1982) *Plant Physiol.* **70**, 39–45

8. Renosto, F., Martin, R. L., Borrell, J. L., Nelson, D. C., and Segel, I. H. (1991) *Arch. Biochem. Biophys.* **290**, 66–78

9. Foster, B. A., Thomas, S. M., Mahr, J. A., Renosto, F., Patel, H., and Segel, I. H. (1994) *J. Biol. Chem.* **269**, 28583–28589

10. MacRae, I., Rose, A. B., and Segel, I. H. (1998) *J. Biol. Chem.* **273**, 28583–28589

11. MacRae, I. J., Segel, I. H., and Fisher, A. J. (2000) *Biochemistry* **39**, 1613–1621

12. Satishchandran, C., Hickman, Y. N., and Markham, G. D. (1992) *Biochemistry* **31**, 11684–11688

13. Monod, J., Wyman, J., and Changeux, J.-P. (1965) *J. Mol. Biol.* **12**, 88–118

14. Segel, I. H. (1993) *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*. pp. 421–464, Wiley-Interscience, New York

15. Ballio, A., Chain, E. B., Dentice di Accadia, F., Navizio, F., Rossi, C., and Ventura, M. T. (1959) *Sel. Sci. Papers Istituto Superiore Sanita* **2**, 343–353

16. Itagashi, M. (1961) *J. Biochem. (Tokyo)* **50**, 52–61

17. Renosto, F., and Segel, I. H. (1977) *Arch. Biochem. Biophys.* **180**, 416–428

18. Hanson, A. D., Rathinasabapathi, B., Chamberlin, B., and Gage, D. A. (1991) *Plant Physiol.* **97**, 1199–1205

19. Ryan, L. D., and Vestling, C. S. (1974) *Arch. Biochem. Biophys.* **160**, 279–284

20. MacRae, I. J., and Segel, I. H. (1992) *Biochemistry* **31**, 66–78

21. Tweedie, J. W., and Segel, I. H. (1971) *Prep. Biochem.* **1**, 91–117

22. Segel, I. H., Renosto, F., and Seubert, A. P. (1987) *Methods Enzymol.* **143**, 334–349

23. Renosto, F., Seubert, P. A., and Segel, I. H. (1984) *J. Biol. Chem.* **259**, 2113–2123

24. Seubert, P. A., Haung, L., Renosto, F., and Segel, I. H. (1983) *Arch. Biochem. Biophys.* **225**, 679–691

25. Venkatachalam, K. V., Fuda, H., Koonin, E. V., and Strutt, C. A. (1999) *J. Biol. Chem.* **274**, 2601–2604

26. Deyrup, A. T., Singh, B., Krishnan, S., Lyle, S., and Schwartz, N. B. (1999) *J. Biol. Chem.* **274**, 28929–28936

27. Rubin, M. M., and Changeux, J.-P. (1966) *J. Mol. Biol.* **11**, 674–683

28. Renosto, F., Seubert, P. A., Knudson, P., and Segel, I. H. (1985) *J. Biol. Chem.* **260**, 1535–1544

29. Pettigrew, D. W., and Frieden, C. (1977) *J. Biol. Chem.* **252**, 4546–4551

30. Wood, H. G., Davis, J. J., and Lochmüller, H. (1966) *J. Biol. Chem.* **241**, 5692–5704

31. Storer, A. C., and Cornish-Bowden, A. (1976) *Biochem. J.* **159**, 1–5