Comparative Stability and Catalytic and Chemical Properties of the Sulfate-Activating Enzymes from *Penicillium chrysogenum* (Mesophile) and *Penicillium duponti* (Thermophile)

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ATP sulfurylases from *Penicillium chrysogenum* (a mesophile) and from *Penicillium duponti* (a thermophile) had a native molecular weight of about 440,000 and a subunit molecular weight of about 69,000. (The *P. duponti* subunit appeared to be a little smaller than the *P. chrysogenum* subunit.) The *P. duponti* enzyme was about 100 times more heat stable than the *P. chrysogenum* enzyme; $k_{\text{inact}}$ (the first-order rate constant for inactivation) at 65°C = $3.3 \times 10^{-2}$ s$^{-1}$ for *P. duponti* and $3.0 \times 10^{-4}$ s$^{-1}$ for *P. chrysogenum*. The *P. duponti* enzyme was also more stable to low pH and urea at 30°C. Rabbit serum antibodies to each enzyme showed heterologous cross-reaction. Amino acid analyses disclosed no major compositional differences between the two enzymes. The analogous $K_m$ and $K_i$ values of the forward and reverse reactions were also essentially identical at 30°C. At 50°C, the physiologically important adenosine 5'-phosphosulfate (APS) synthetase activity of the *P. duponti* enzyme was 4 U mg of protein$^{-1}$, which is about half that of the *P. chrysogenum* enzyme. The molybdohydrolase and ATP synthetase activities of the *P. duponti* enzyme at 30°C were similar to those of the *P. chrysogenum* enzyme. At 50°C, the APS synthetase activity of the *P. duponti* enzyme was 12 to 19 U mg of protein$^{-1}$, which was higher than that of the *P. chrysogenum* enzyme at 30°C (8 ± 1 U mg of protein$^{-1}$). Treatment of the *P. chrysogenum* enzyme with 5,5'-dithiobis(2-nitrobenzoate) (DTNB) at 30°C under non-denaturing conditions modified one free sulfhydryl group per subunit. $V_{\text{max}}$ was not significantly altered, but the catalytic activity at low magnesium-ATP or $\mathrm{SO}_4^{2-}$ (or $\mathrm{MoO}_4^{2-}$) was markedly reduced. Chemical modification with tetrantitromethane had the same results on the kinetics. The native *P. duponti* enzyme was relatively unreactive toward DTNB or tetrantitromethane at 30°C and pH 8.0 or pH 9.0, but at 50°C and pH 8.0, DTNB rapidly modified one SH group per subunit. APS kinase (the second sulfate-activating enzyme) of *P. chrysogenum* dissociated into inactive subunits at 42°C. The *P. duponti* enzyme remained intact and active at 42°C.

Thermophilic microorganisms provide a unique opportunity to study structure-function relationships of selected enzymes. By comparing the properties of an enzyme isolated from a mesophile with those of the same enzyme isolated from a closely related thermophile, several interesting questions can be answered. For example: (i) Is the thermophile enzyme more stable to heat denaturation? Or does the thermophile compensate for a more rapid rate of enzyme denaturation by an increased rate of enzyme synthesis? (ii) If the thermophile enzyme is more stable (and usually, this is the case), what structural features account for the increased stability? (iii) Are the kinetic and catalytic properties of the thermophile enzyme particularly well adapted to operate at elevated temperatures? (iv) Must the thermophile enzyme sacrifice catalytic power to gain increased thermal stability? During the past several years, we have explored some of these areas by comparing the ATP sulfurylase (ATP:sulfate adenylyltransferase [EC 2.7.7.4]) and adenosine 5'-phosphosulfate (APS) kinase (ATP:APS 3'-phosphotransferase [EC 2.7.1.25]) from *Penicillium chrysogenum* with the same enzymes from *Penicillium duponti* (also called *Talaromyces duponti* [1]). These enzymes catalyze, respectively, the first two reactions in the assimilation of inorganic sulfate. The *P. chrysogenum* used in our studies is a typical wild-type strain which grows well on synthetic medium at 15 to 30°C but poorly or not at all at 37°C. *P. duponti* is a true thermophile which grows poorly or not at all at 25°C, has an optimum growth temperature of about 47 to 50°C, and has a maximum growth temperature of about 57 to 60°C.

In this paper, we report some of our comparative studies on chemical composition, enzyme stability, and kinetic properties.

**MATERIALS AND METHODS**

**Organisms and culture conditions.** The two organisms used in this study were *P. chrysogenum* (mesophile; ATCC 24791; called strain PS-75 in some earlier publications) and *P. duponti* (thermophile). The *P. duponti* strain was obtained from T. Woodin, University of Nevada, Reno. Both organisms were routinely maintained on tomato juice agar (600 ml of commercial tomato juice diluted with 400 ml of water and solidified with 3% agar). Mycelial cultures were grown in shaken flasks or 10-liter New Brunswick fermentor jars. The synthetic medium (initially at pH 7) contained (per liter): 10 g of ammonium citrate (dibasic), 6 g of ammonium phosphate (dibasic), 16 g of potassium phosphate (dibasic), 0.1 g of l-cysteic acid (sole sulfur source), 10 ml of trace metals solution (22), and 40 g of commercial cane sucrose. The sucrose was sterilized separately as a 40% (wt/vol) solution and added to the rest of the medium just before inoculation. The *P. duponti* medium was supplemented with 0.5 mg of biotin per liter. For enzyme production, 100 ml of a 24-h-old cell suspension was transferred to a 3-liter...
Fernbach flask containing 900 ml of medium. The new culture was used after 24 h of growth to inoculate a New Brunswick Microferm fermentor jar containing 9 liters of prewarmed medium. The culture was then incubated for 24 h at 22 to 25°C (P. chrysogenum) or 50°C (P. duponti) with an air flow of 9 liters min⁻¹ and an agitator speed of 200 rpm (P. chrysogenum) or 14 liters min⁻¹ and 400 rpm (P. duponti). After 24 h, the total mycelial yields were about 300 to 500 g (wet weight) (P. chrysogenum) and 200 to 400 g (wet weight) (P. duponti).

**Purification of ATP sulfurylase and APS kinase.** The 24-h-old mycelium was collected by suction filtration through four layers of cheesecloth and washed several times with either ice-cold deionized water (P. chrysogenum) or room-temperature 0.05 M Tris chloride buffer, pH 8.0 (P. duponti). The washed and blotted mycelial pad was frozen in small batches with liquid nitrogen and then ground to a fine powder in a Waring blender. The frozen powder was stirred into 1,200 ml of 0.3 M Tris chloride (pH 8.0) containing 5 mM EDTA at room temperature. After thawing, the homogenate was centrifuged at 15,000 × g for 10 min (5°C).

ATP sulfurylase from P. chrysogenum was purified by minor modifications of methods described earlier (5, 19) with the omission of the heat step. Briefly, the procedure included (in order) ammonium sulfate precipitation (30 to 55% pellet), blue dextran-Sepharose chromatography (0 to 1 M linear NaCl gradient), DEAE-cellulose chromatography (0 to 1 M linear NaCl gradient), and gel filtration (Bio-Gel agarose A-1.5m). All steps were carried out at 5°C. The final preparation was stored as a frozen concentrated solution (0.3 to 0.5 mg ml⁻¹) in 0.04 M Tris chloride buffer (pH 8.0) (pH measured at room temperature). Partially purified or older (partially inactivated) homogeneous preparations were used for stability studies. These preparations generally had a standard molybdolyis assay activity (5, 13) of 16 ± 1 U mg of protein⁻¹. Fresh homogeneous preparations (standard molybdolyis activity, 21 ± 3 U mg of protein⁻¹) were used for kinetics studies and amino acid analyses. ATP sulfurylase from P. duponti was prepared by a similar procedure with the following modifications. (i) All column steps except the gel filtration on A-1.5m were carried out at room temperature (ca. 23°C); (ii) the protease phenylmethylsulfonyl fluoride (ca. 3 mg liter⁻¹, final concentration) was included in all solutions; (iii) a 30 to 60% ammonium sulfate cut was taken at 25°C (7); (iv) Affi-Gel blue was substituted for the homemade blue dextran in the first column chromatography step; and (v) a final Matrix Gel Green A (Amicon) adsorption-NaCl elution step was added.

APS kinase from P. chrysogenum was purified as described previously (16). The same procedure was used to prepare the P. duponti enzyme, but the final preparation was not homogeneous or particularly stable under the storage conditions used for the P. chrysogenum enzyme. However, the enzyme activity obtained was sufficient for a brief comparative heat stability study.

**Enzyme assays.** In studies of temperature effects on activity and stability, ATP sulfurylase was measured by the P₁ colorimetric molybdolyis assay (5, 13). Most kinetics studies employed the continuous, coupled-enzyme spectrophotometric assays described previously (16, 18). The commercial coupling enzymes were desalted by gel filtration (Sephadex G-25) before use. The kinetic constants of the reverse reaction (Kₘₗ, Kᵢ, and Kₘᵢ) were determined by the ³⁵SO₄²⁻ release assay described previously (18a). One unit of activity is the amount of enzyme which catalyzes the formation of 1 μmol of primary product in 1 min.

**Protein determination.** Protein concentrations of purified ATP sulfurylase preparations were determined from the A₂₇₅ (20): [protein] (mg/ml) = A₂₇₅/0.871. The calculation assumes that the specific absorbivity of the P. duponti enzyme is the same as that of the P. chrysogenum enzyme.

**Amino acid analyses.** Pure desalted ATP sulfurylases were concentrated with an Amicon membrane filtration device and washed on the filter with deionized water to remove traces of buffer. The enzyme (20- to 35-μg quantities) was hydrolyzed in 6 N HCl at 110°C for 24, 48, and 72 h. A separate sample of each enzyme was treated with performic acid for 20 h and then hydrolyzed in 6 N HCl at 110°C for 24 h. Amino acid analyses were performed on a Beckman model 6300 amino acid analyzer by Al Smith of the Protein Structure Laboratory of University of California, Davis.

**Antibody production.** Male New Zealand rabbits were injected subcutaneously with ca. 1 mg of pure enzyme in 0.9% NaCl containing complete Freund adjuvant. Injections were repeated after 7, 19, and 27 days. After 30 days, 3 ml of blood was obtained from the marginal ear vein, and the cell-free serum was fractionated with Na₂SO₄ and then dialyzed against NaCl-borate buffer (10). The dialyzed solutions were stored at -20°C. Double-diffusion tests were performed by the method of Ouchterlony (14). The agar plates contained 0.9% agar, 2% NaCl, and 5 mM disodium EDTA at pH 7.5.

**RESULTS**

**Native and subunit molecular weights.** ATP sulfurylase from both sources eluted from a calibrated Bio-Gel A-1.5m column just before ferritin at a position corresponding to a native molecular weight around 440,000. Co-gel filtration of equal amounts of the two enzymes yielded a single, nearly symmetrical activity profile. After heat inactivation of the thermolabile (P. chrysogenum) component, the peak of remaining activity was at the same position as the peak of combined activity (Fig. 1). Sodium dodecyl sulfate-gel electrophoresis (8) of 1- to 10-μg quantities of the most highly purified preparations in 12% acrylamide revealed, in each case, a single dye-staining band. Compared with standards of known molecular weight, the mobility of the P. chrysogenum subunit corresponded to a molecular weight of 69,000 ± 1,000. The P. duponti subunit migrated at a slightly more rapid rate suggesting a slightly lower molecular weight. Clearly, the two enzymes are remarkably similar in native size and subunit composition. Both appear to be a hexamer composed of equal-size subunits.

**Amino acid analysis.** The P. chrysogenum and P. duponti ATP sulfurylases had nearly identical amino acid compositions and relative abundances of residues (Table 1). The similarity is not surprising given the size of the enzyme (subunit molecular weight, 69,000; ca. 550 amino acid residues).

**Immunological cross-reaction.** Antisera to each enzyme cross-reacted with the heterologous enzyme (Fig. 2), indicating similar structural determinants. The spurs show that the two enzymes are not exactly identical.

**Influence of temperature on ATP sulfurylase activity.** Figure 3 shows the activity-temperature profiles and the corresponding Arrhenius plots for the P. chrysogenum and P. duponti enzymes. The E₅₀ values are in the usual ranges for enzyme activity (see p. 941 of reference 17). For a 10-min assay period, the optimum temperatures of the two enzymes differed by about 20°C.

**Effect of temperature on enzyme inactivation.** Both enzymes underwent a first-order inactivation at elevated tem-
from the P. duponti analogous the P. chrysogenum enzyme. Figure 4. Inactivation was irreversible; prolonged storage of the heat-treated enzymes at 0 or 30°C failed to recover activity. Figure 5 graphically illustrates the difference between the heat stabilities of the two enzymes. For example, at 65°C, the P. chrysogenum enzyme denatured with a rate constant nearly 100-fold greater than that of the P. duponti enzyme. The Arrhenius plots for inactivation are linear and yield $E_a$ values of $>100,000$ cal ($>4.2 \times 10^5$ J) mol$^{-1}$. Activation energy constants for denaturation are summarized in Table 2. The more positive $\Delta S^\circ$ for inactivation of the P. duponti enzyme is consistent with a tighter or more highly ordered native state compared with that of the P. chrysogenum enzyme.

**pH and urea stability of ATP sulfurylases.** Heat-stable proteins usually exhibit increased stability toward other denaturants as well. P. duponti ATP sulfurylase was no exception (Fig. 6). At pH 4.36, the P. chrysogenum enzyme was inactivated at a rate 100 times greater than that of the P. duponti enzyme. Reading Fig. 6 differently, we see that in comparison with the P. chrysogenum enzyme, the P. duponti enzyme requires a pH that is 0.6 to 0.8 unit lower for the same inactivation rate.

**Kinetic properties of ATP sulfurylase at 30°C.** Table 3 summarizes the kinetic constants of the ATP sulfurylases from the two fungi. At 30°C, the P. duponti enzyme had somewhat lower $V_{max}$ values for all reactions examined, but the analogous $K_m$ and $K_V$ values of the two enzymes were almost identical. It is noteworthy that the $V_{max}$ of the P. duponti enzyme with sulfate as the inorganic substrate (the physiologically important reaction) was only about 50% of the corresponding $V_{max}$ of the P. chrysogenum enzyme but that the $V_{max}$ of the P. duponti enzyme with molybdate was almost the same as that of the P. chrysogenum enzyme. The

### Table 1. Approximate amino acid composition of ATP sulfurylases

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>P. chrysogenum</th>
<th>P. duponti</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate$^a$</td>
<td>10.0</td>
<td>11.1</td>
</tr>
<tr>
<td>Aspartate$^a$</td>
<td>9.7</td>
<td>9.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>9.6</td>
<td>8.9</td>
</tr>
<tr>
<td>Leucine</td>
<td>9.6</td>
<td>10.4</td>
</tr>
<tr>
<td>Arginine</td>
<td>8.5</td>
<td>9.2</td>
</tr>
<tr>
<td>Glycine</td>
<td>7.4</td>
<td>7.9</td>
</tr>
<tr>
<td>Valine</td>
<td>7.1</td>
<td>7.6</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.7</td>
<td>5.3</td>
</tr>
<tr>
<td>Proline</td>
<td>5.3</td>
<td>5.2</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.9</td>
<td>4.1</td>
</tr>
<tr>
<td>Serine</td>
<td>4.5</td>
<td>3.9</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.2</td>
<td>3.8</td>
</tr>
<tr>
<td>Lysine</td>
<td>3.9</td>
<td>4.7</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.5</td>
<td>4.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.3</td>
<td>3.0</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.3</td>
<td>0.7</td>
</tr>
<tr>
<td>Tryptophan$^b$</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>Cysteine</td>
<td>ca. 0.6</td>
<td>ca. 0.4</td>
</tr>
</tbody>
</table>

$^a$ The values include the corresponding amides.

$^b$ Determined by the method of Edelhoch (2) except that the guanidine hydrochloride concentration was 3.4 M instead of 6 M.

**FIG. 2.** Antibody-enzyme cross-reaction. Upper left: Antibodies to P. duponti ATP sulfurylase in center well; alternating enzymes in peripheral wells. Upper right: Antibodies to P. duponti enzyme in center well; P. duponti enzyme in all peripheral wells. Lower left: Antibodies to P. chrysogenum ATP sulfurylase in center well; alternating enzymes in peripheral wells. Lower right: Antibodies to P. chrysogenum enzyme in center well; P. chrysogenum enzyme in all peripheral wells. In the alternating enzyme series, the peripheral wells at the 2, 6, and 10 o'clock positions contained 12 µg of the homologous enzyme; the wells at 12, 4, and 8 o'clock contained 12 µg of the heterologous enzyme.
VOL.
The assay of pyrophosphatase was converted to temperature. The reaction with molybdate short-circuits with prepared chrysogenum enzyme to molybdolysis.

FIG. 3. Variation in catalytic (molybdolysis) activity as a function of assay temperature and corresponding Arrhenius plots of activity for ATP sulfurylase from P. chrysogenum and P. duponti. The assay buffer was adjusted to pH 8.0 at each experimental temperature. The assay time was 10 min. (The PPi formed was converted to Pi in a postreaction incubation with inorganic pyrophosphatase at 30°C after inactivating ATP sulfurylase by a 1-min heating in a boiling water bath.) T, Temperature.

difference may reflect the greater ability of the P. chrysogenum enzyme to form or release APS at 30°C. (The reaction with molybdate short-circuits the overall catalytic process; the products are AMP, PPi, and MoO4^{2-}. Compared with APS, AMP has very little affinity for the enzyme.)

Specific activities of P. duponti ATP sulfurylase at 50°C. A complete kinetics study of the P. duponti enzyme at high temperatures could not be performed because the necessary heat-stable coupling enzymes were not available. However, the molybdolysis activity (via the Pi colorimetric assay) and the reverse reaction activity with APS plus PPi (via the 35SO4^{2-} release assay) could be measured at high temperature. For the homogenous P. duponti enzyme at 50°C, Vmax, (MoO4^{2-}) was 96 U mg of protein^{-1}; Vmax, was 150 U mg of protein^{-1}. At 30°C, the ratios of Vmax, (SO4^{2-})/Vmax, (MoO4^{2-}) and Vmax, (SO4^{2-})/Vmax, (APS plus PPi) were about 0.2 and 0.08, respectively. If the same ratios hold at 50°C, then Vmax, (SO4^{2-}) would be about 12 to 19 U mg of protein^{-1}, which is greater than Vmax, (SO4^{2-}) for the P. chrysogenum enzyme at 30°C.

In an attempt to measure Vmax, (SO4^{2-}) at 50°C directly, we used the Pi colorimetric assay. The incubation mixture contained 10 mM magnesium-ATP, 10 mM SO4^{2-}, 5 mM excess Mg^{2+}, 10 U of inorganic pyrophosphatase ml^{-1}, 0.5 U of APS kinase ml^{-1}, and 2.5 μg of ATP sulfurylase ml^{-1} in 0.05 M Tris chloride buffer, pH 8.0. The reaction was started by the simultaneous addition of the three enzymes. This assay is not particularly accurate because of the heat lability.
of the pyrophosphatase and (especially) APS kinase (15) and the resulting product inhibition exerted by APS (18). Nevertheless, measurements of PP, produced at 15, 30, and 45 s yielded a minimum specific activity (Vmax) of about 20 U mg of protein⁻¹. In another series of experiments, Vmax (SO₄²⁻) was determined at 21 and 30°C. Eₚ calculated from the Arrhenius equation (see p. 932 of reference 17) was 12,780 cal (53,472 J) mol⁻¹ (about 1 kcal [4 kJ] mol⁻¹) higher than with MoO₄²⁻ as the substrate). Using this Eₚ, Vmax at 50°C was calculated to be 14.3 U mg of protein⁻¹.

Chemical modification by group-specific reagents. ATP sulfurylase from P. chrysogenum reacted with 5,5'-dithiobis(2-nitrobenzoate) (DTNB; Ellman reagent), indicating the presence of one reactive sulfhydryl group per subunit (Fig. 7). The half-time for the reaction at 30°C, pH 8.0, and 25 μM DTNB was about 2.6 min (k = 0.27 min⁻¹). Except for what might be a short lag (or experimental error), the semilog plot of (A₀ - A) versus time (inset of Fig. 7) is linear, indicating that all available SH groups (six per native enzyme) have essentially the same intrinsic reactivity and that modification of one group has little if any effect on the reactivity of the others. Tweedie and Segel (20) and Farley et al. (4) reported that sulfhydryl modification had no effect on the activity of the enzyme. But in both of these earlier studies, catalytic activity was measured at saturating magnesium-ATP and molybdate. Figure 8 shows the effect of sulfhydryl modification on the initial velocity of the P. chrysogenum enzyme with sulfate as the inorganic substrate. Treatment with DTNB had very little effect on Vmax but severely depressed activity at unsaturating substrate levels. In fact, the reciprocal plots for the modified enzyme were nonlinear (concave up), suggesting cooperative interactions which are not observed with the native enzyme. The v versus [S] plots were sigmoidal. Hill plots yielded an nH of about 2 for both substrates.) Activity measured with molybdate yielded essentially the same results. Overnight incubation of the DTNB-modified enzyme with 5 mM β-mercaptoethanol at 5°C restored 92% of the catalytic activity measured at 5 mM magnesium-ATP and 0.1 mM molybdate.

Tetranitromethane (TNM) had an effect identical to that of DTNB (Fig. 8) except that <4% recovery of activity was achieved by overnight incubation with β-mercaptoethanol. Farley et al. (4) concluded that the TNM target group was a tyrosyl side chain because of the apparent ineffectiveness of DTNB. Indeed, TNM does modify tyrosyl groups on the enzyme, but it now seems likely that TNM affected enzyme activity by oxidizing the sulfhydryl group (4).

Only a very slow reaction between DTNB and the native P. duponti enzyme could be detected spectrophotometrically at 30°C and pH 8.0 (Fig. 7) or pH 9.0 (data not shown). A 30-min preincubation with 25 μM DTNB had little effect on enzyme activity measured at high or low substrate concentrations. TNM (20 μM) was similarly without significant effect on the P. duponti enzyme at low temperature. However, at 50°C and pH 8.0, the P. duponti enzyme reacted rapidly with DTNB; 4.1 μM enzyme sites liberated 4 μM thionitrobenzoate (data not shown). The half-time for modification was about 2 min (k = 0.35 min⁻¹). The modified enzyme showed the same altered kinetics when assayed at 30°C as that displayed by the P. chrysogenum enzyme.

In addition to the readily modified SH group, the P.
SULFATE-ACTIVATING ENZYMES FROM *PENICILLIUM* SPP.

**FIG. 6.** Effect of pH and urea concentration on the inactivation rate constant. The $k$ values for pH inactivation were estimated from the initial slopes of the semilog plots which were nonlinear. T, Temperature; $t_{1/2}$, half-life.

*chrysogenum* enzyme subunit contains two other SH groups which become accessible to DTNB in the presence of guanidine hydrochloride or sodium dodecyl sulfate (data not shown). Under the same denaturing conditions at 30°C, a total of two SH groups in the *P. duponti* enzyme could be modified by DTNB. Presumably, one of them is the SH that can be modified under non-denaturing conditions at 50°C. It is noteworthy that as little as 0.01% (wt/vol) sodium dodecyl sulfate was sufficient to expose the buried SH groups of the *P. chrysogenum* enzyme. In contrast, the *P. duponti* enzyme required 0.03% sodium dodecyl sulfate before any reaction with DTNB occurred. The total cysteine content of each enzyme was quite low (Table 1), and consequently, the amino acid analyses may not be entirely accurate. Taking the analyses at face value, it appears that the free SH groups account for all (or nearly all) of the cysteine residues (i.e., there may be no disulfide linkages in either enzyme).

Phenyglyoxal, a reagent generally considered to be specific for arginyl side chains (6, 12), has been shown to also modify sulphydryl groups in some enzymes (3). The native *P. chrysogenum* enzyme (4 μM sites) was preincubated for 60 min with 13.4 mM phenylglyoxal at 30°C and pH 8.0 in the presence of 50 mM borate. A subsequent addition of 25 μM DTNB resulted in the formation of 4 μM thionitrobenzoate. The result indicates that phenylglyoxal did not react with the accessible SH group of ATP sulfurylase. However, the activity of the phenylglyoxal-modified enzyme measured at 10 mM magnesium-ATP and 10 mM MoO$_4^{2-}$ was decreased. A similar inactivation of the *P. duponti* enzyme at 30°C was observed. It is likely then that both enzymes contain at least one essential arginyl side chain.

APS kinase. APS kinase from *P. chrysogenum* loses activity at temperatures >35°C. When the heat-inactivated enzyme is cooled and stored at 0 to 30°C, activity reappears.
### TABLE 3. Kinetic constants of ATP sulfurylases at 30°C

<table>
<thead>
<tr>
<th>Constant</th>
<th>Description</th>
<th>Value with the following enzyme source:</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$</td>
<td>Maximum forward velocity with sulfate</td>
<td>$8 \pm 1$ U mg of protein$^{-1}$</td>
</tr>
<tr>
<td>$K_{m}$</td>
<td>Maximum forward velocity with molybdate</td>
<td>$21 \pm 3$ U mg of protein$^{-1}$</td>
</tr>
<tr>
<td>$K_m^{m}$</td>
<td>$K_m$ for magnesium-ATP at saturating sulfate</td>
<td>$0.18$ mM</td>
</tr>
<tr>
<td>$K_m^{s}$</td>
<td>$K_m$ for molybdate at saturating magnesium-ATP</td>
<td>$0.05$ mM</td>
</tr>
<tr>
<td>$K_i$</td>
<td>Dissociation constant of $E \cdot$ magnesium-ATP (from kinetic measurements)</td>
<td>$0.42, 0.67$ mM</td>
</tr>
<tr>
<td>$K_m^{m}$</td>
<td>$K_m$ for sulfate at saturating magnesium-ATP</td>
<td>$0.55$ mM</td>
</tr>
<tr>
<td>$K_m^{s}$</td>
<td>$K_m$ for molybdate at saturating magnesium-ATP</td>
<td>$0.11$ mM</td>
</tr>
<tr>
<td>$K_{iso}$</td>
<td>Inhibition constant of $SO_4^{2-}$ (dissociation constant of $E \cdot$ magnesium-ATP $\cdot$ $SO_4^{2-}$)</td>
<td>$3.4$ μM</td>
</tr>
<tr>
<td>$K_{m}$</td>
<td>Inhibition constant of $HPO_4^{2-}$ (dissociation constant of $E \cdot$ $HPO_4^{2-}$ and magnesium-ATP $\cdot$ $HPO_4^{2-}$)</td>
<td>$0.36$ mM</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>Maximum reverse velocity</td>
<td>$60$ U mg of protein$^{-1}$</td>
</tr>
<tr>
<td>$K_m^{m}$</td>
<td>$K_m$ for APS at saturating $PP_i$</td>
<td>$0.3$ μM</td>
</tr>
<tr>
<td>$K_i$</td>
<td>Dissociation constant of $E \cdot$ APS (kinetic measure)</td>
<td>$0.04$ μM</td>
</tr>
<tr>
<td>$K_m^{m}$</td>
<td>$K_m$ for $PP_i$ (total species) at saturating APS</td>
<td>$6.5$ μM</td>
</tr>
<tr>
<td>$V_{max}^{SO_4^{2-}}/V_{max}^{(MoO_4^{2-})}$</td>
<td>Ratio of $V_{max}$ values with sulfate and molybdate as inorganic substrates</td>
<td>$0.32$</td>
</tr>
<tr>
<td>$V_{max}^{SO_4^{2-}}/V_{max}^{(PP_i)}$</td>
<td>Ratio of $V_{max}$ with sulfate to $V_{max}$ with APS + $PP_i$</td>
<td>$0.13$</td>
</tr>
</tbody>
</table>

$^a$ All constants were determined at pH 8.0 and 30°C in the presence of 5 mM excess Mg$^{2+}$. Continuous, coupled-enzyme spectrophotometric assays were used except in the determination of $K_i$ and $K_{iso}$. These constants were determined by the more sensitive $35SO_4^{2-}$ release assay (18a).

$^b$ Enzyme concentrations used for specific activity calculations were based on a specific absorbivity of 0.871 at 278 nm (19) for both enzymes.

$^c$ Determined with sulfate and with molybdate as the inorganic substrate. The different values for any one organism provide an indication of experimental error because $K_m$ should be constant and independent of the inorganic substrate.

Several lines of evidence suggest that the reversible inactivation results from subunit dissociation-reassociation (15). Gel filtration of the *P. chrysogenum* enzyme at 22°C yielded a single activity peak which eluted from the column at a position corresponding to a molecular weight of 57,000. At 46°C, no APS kinase activity was found in the eluate, but after overnight incubation at 22°C, activity was found in the fraction corresponding to a molecular weight of 33,000 (Fig. 9). *P. duponti* APS kinase activity eluted at the same position (molecular weight, ca. 57,000) at 22 or 46°C (data not shown). Co-gel filtration of the *P. chrysogenum* and *P. duponti* enzymes yielded a single activity peak at 22°C, but at 44°C, and *P. chrysogenum* enzyme (assayed after reactivation) eluted after the active *P. duponti* enzyme (Fig. 10).

### DISCUSSION

ATP sulfurylase from the thermophilic fungus *P. duponti* was essentially identical in size, subunit composition, and amino acid composition to the same enzyme from the mesophilic *P. chrysogenum*. Except for $V_{max}$ values, the two enzymes were kinetically similar at 30°C. However, the *P. duponti* enzyme was considerably more stable to common denaturing agents (heat, low pH, urea). Antibody cross-reaction patterns confirmed that the two enzymes have homologous structural regions although they are not completely identical.

At 30°C, the *P. duponti* enzyme had a lower specific activity than the *P. chrysogenum* enzyme for the physiologically important reaction of APS synthesis. This is consistent with the suggestion that to have the flexibility required for maximum catalytic activity at optimal temperature, a thermophile enzyme will show decreased catalytic activity at...
lower temperatures compared with the same enzyme from a mesophile (11, 21, 23).

At 30°C, homogeneous *P. duponti* ATP sulfurylase had specific activities of 12 to 19 and 96 U mg of protein⁻¹ with sulfate and molybdate, respectively, as the inorganic substrate. In comparison, the specific activities of the *P. chrysogenum* enzyme with these two substrates at 30°C (ca. the optimal temperature for this organism) were 7 to 9 and 18 to 24 U mg of protein⁻¹, respectively. Thus, no catalytic power had to be sacrificed by the *P. duponti* enzyme to form a more stable structure. Indeed, one might conclude that the *P. duponti* enzyme is a better catalyst than the *P. chrysogenum* enzyme. The thermophile enzyme not only has a markedly greater thermal stability, but it also has a higher APS synthesis activity at the optimal temperature of the organism. In any event, such comparisons are not always as informative as they appear. The optimal temperature for growth of an organism is not necessarily the physiologically relevant temperature at which an organism spends most of its life in nature, or even the environmental temperature at which the product of the specific enzyme catalyzed reaction plays an indispensible role. It is this latter temperature that would exert the major selective pressure on protein evolution.

Although the cellular level of ATP sulfurylase in each organism is not known, the yield of enzyme after the first column step was always lower for the *P. duponti* preparation. It is possible that the thermophile capitalizes on the greater APS synthesis activity of its ATP sulfurylase at optimal temperature by producing less of the enzyme.

Native ATP sulfurylases from both sources contained a single reactive sulphydryl group per subunit. Chemical modification of this group resulted in a dramatic increase in [S]₀₉₅ for both substrates. At 30°C and pH 8.0 or pH 9.0, the *P. duponti* enzyme SH group was relatively unreactive. Increasing the temperature at constant pH markedly increased the reactivity of the group. The effect of temperature probably reflects a conformational change which exposes the previously buried SH group and increases its degree of ionization. (The DTNB-reactive species is the RS⁻ form.) The function of the SH group is unknown. It clearly is not involved in catalysis because modification had little or no effect on Vₘₐₓ (i.e., Kₐ₅). It is tempting to speculate about a potential regulatory role for the SH group. After all, chemical modification of the group had a result identical to that of a "K-type" allosteric inhibitor. A reversible SH modification process by (e.g.) methylation-demethylation or oxidation-reduction via disulfide interchange (9) would provide an effective on-off switch with respect to catalytic activity at unsaturating substrate levels. It is also conceivable that in vitro SH modification merely induces the same conformational change in the enzyme that is normally triggered in vivo by a natural reversibly bound effector or by a more common type of covalent modification (phosphorylation, adenylation).

Most considerations of thermophile enzyme stability have

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**FIG. 8.** Initial velocity kinetics of *P. chrysogenum* ATP sulfurylase which had been previously modified by 50 μM DTNB or 20 μM TNM for 30 min. In the substrate concentration range studied, the control plots would appear to be nearly horizontal (ES is ≈Kₑ₅₅).
been concerned with tertiary structure. Our preliminary study of the *P. duponti* APS kinase suggests that thermophile enzymes may also show increased quaternary structure stability.

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LITERATURE CITED


FIG. 10. Co-gel filtration of APS kinase from *P. chrysogenum* (mesophile) and *P. duponti* (thermophile). Elution profiles for the enzymes from *P. duponti* (A) and *P. chrysogenum* (B) at 44°C and for a mixture of the two enzymes at 22°C (C) and 44°C (D). The conditions were the same as those described in the legend to Fig. 9. In the experiments shown in panels C and D, the *P. duponti* enzyme was assayed at 44°C after preincubation to inactivate *P. chrysogenum* enzyme. In the experiment shown in panel C, the *P. chrysogenum* enzyme was assayed at 25°C. The *P. duponti* enzyme has negligible activity at this temperature. In the experiment shown in panel D, the *P. chrysogenum* enzyme was assayed after the eluates were stored at 0°C overnight.
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