ATP Sulfurylase from *Penicillium chrysogenum*

MOLECULAR BASIS OF THE SIGMOIDAL VELOCITY CURVES INDUCED BY SULFHYDRYL GROUP MODIFICATION

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ATP sulfurylase from *Penicillium chrysogenum* is a noncooperative homooligomer containing three free sulfhydryl groups per subunit. Under nondenaturing conditions, one SH group per subunit was modified by 5,5'-dithiobis-(2-nitrobenzoate), or N-ethylmaleimide. Modification had only a small effect on *k*_m, but markedly increased the [S]_0.5 values for the substrates, MgATP and SO_4^-2. MgATP and adenosine-5'-phosphosulfate protected against modification. The SH-modified enzyme displayed sigmoidal velocity curves for both substrates with Hill coefficients (n_H) of 2. Fluorosulphonate (FSO^-) and other dead-end inhibitors competitive with SO_4^-2 activated the SH-modified enzyme at low SO_4^-2 concentration. In order to determine whether the sigmoidality resulted from true cooperative binding (as opposed to a kinetically based mechanism), the shapes of the binding curves were established from the degree of protection provided by a ligand against phenylglyoxal-dependent irreversible inactivation under noncatalytic conditions. Under standard conditions (0.05 M Na-N-(2-hydroxyethyl)piperezine-N'-3-propanesulfonic acid buffer, pH 8, 30 °C, and 3 mM phenylglyoxal), the native enzyme was inactivated with a *k* of 2.67 ± 0.25 × 10^-3 s^-1, whereas *k* for the SH-modified enzyme was 5.44 ± 0.27 × 10^-3 s^-1. The increased sensitivity of the modified enzyme resulted from increased reactivity of ligand-protectable groups. Both the native and the SH-modified enzyme displayed hyperbolic plots of ∆k (i.e., protection) versus [MgATP], or [FSO^-], or [SO_4^-2] in the absence of coligand (n_H = 0.98 ± 0.06). The plots of ∆k versus [ligand] for the native enzyme were also hyperbolic in the presence of a fixed concentration of coligand. However, in the presence of a fixed [FSO^-] or [SO_4^-2], the ∆k versus [MgATP] plot for the SH-modified enzyme was sigmoidal, as was the plot of ∆k versus [FSO^-] or [SO_4^-2] in the presence of a fixed [MgATP]. The *n*_H values were 1.92 ± 0.09. The results indicate that substrates (or analogs) bind hyperbolically to unoccupied SH-modified subunits, but in a subunit-cooperative fashion to form a ternary complex.

ATP sulfurylase (ATP:sulfate adenylytransferase, EC 3.6.1.1) catalyzes the first reaction in the assimilation of inorganic sulfate: MgATP + SO_4^-2 → MgPP, + APS. The enzyme from *Penicillium chrysogenum* is a homooligomer with a subunit *M* of about 69,000. Each subunit contains three free sulfhydryl groups, one of which can be readily modified under nondenaturing conditions. Tweedie and Segel (1) and later, Farley et al. (2) reported that chemical modification of this SH group had no effect on catalytic activity. However, in these earlier studies, activity was measured at near-saturating substrate concentrations. More recent studies disclosed that sulfhydryl modification markedly affected enzyme activity at subsaturating substrate concentrations (3). The experiments reported in this present paper were designed to characterize the kinetic consequences of SH group modification.

**MATERIALS AND METHODS**

**Enzyme Purification and Assays.**—The purification of ATP sulfurylase and APS kinase, the assay methods (3-10), and the general procedures used to characterize the kinetics of inactivation in the presence and absence of protective ligands (11-12) have been described previously.

Freshly prepared, homogeneous ATP sulfurylase was used in experiments where an accurate knowledge of active site concentration was required (e.g. Fig. 1). The specific activity of the homogeneous enzyme is 21 ± 3 units × mg protein^-1^ in the molybdobis assay and 8 ± 1 units × mg protein^-1^ in the APS synthesis assay. Aged preparations with slightly decreased specific activities were used in some of the kinetic and inactivation experiments.

Protein concentration was determined from the absorbances (1-cm cuvette) at 278 nm (5), 280 and 260 nm (Equation 5-4 of Ref. 13), and 280 and 235 nm (14). A typical stock solution was calculated to contain respectively, 0.97 mg × ml^-1^, 0.96 mg × ml^-1^, and 0.99 mg × ml^-1^, by the above methods.

**Premodification with NEM.**—The enzyme (15 µm sites) was incubated with 150 µM NEM in 0.05 M Na-MOPS, pH 7.0. After 1 h residual NEM was removed and the enzyme transferred to 0.05 M Na-EPPS, pH 8.0, by repeated washing on a Centricon® membrane filter.

**Inactivation by Phenylglyoxal.**—The native or NEM-modified enzyme (0.5 µm sites unless otherwise indicated) was preincubated for various periods in 0.05 M Na-EPPS buffer (pH 8.0, 30 °C) containing 3 mM phenylglyoxal, the protective ligands, and (unless otherwise indicated) 5 mM excess Mg^2+. The incubation volume was 0.22 ml. Periodically, a 20-µl sample was withdrawn and the residual activity measured at 15 mM MgATP, 5 mM excess Mg^2+, and 30 mM SO_4^-2 in a continuous spectrophotometric assay coupled to APS kinase, pyruvate kinase, and lactate dehydrogenase (8). Inactivation rate constants (*k*_inact) were determined from the slopes of log *r*% remaining activity versus time plots. At least three time points were used to determine *k*_inact.

The abbreviations used are: APS, adenosine 5'-phosphosulfate (5'-adenylsulfate); SDS, sodium dodecyl sulfate; DTNB, 5,5'-dithiobis-(2-nitrobenzoate); pCMB, p-chloromercuribenzoate; A-485, acetamidomaleimidylstibine disulfonic acid; NEM, N-ethylmaleimide; MOPS, 3-(N-morpholino)propanesulfonic acid; EPPS, N-(2-hydroxyethyl)piperazine-N'-(3-propanesulfonic acid; MES 2-(N-morpholino)ethanesulfonic acid.
generate the linear semilog plot. In the absence of phenylglyoxal, so inactivation was evident over the experimental incubation periods (<30 min). In sequential quadruplicate measurements made over a 5-h period with the same solution of phenylglyoxal, $k_{app}$ varied by less than ±4% from the mean. The variance in daily replicates using a freshly prepared phenylglyoxal solution each time was greater: $k_{app}$ for the native enzyme averaged $2.7 \times 10^{-4}$ s$^{-1}$ with a maximum deviation of ±0.3 $\times 10^{-4}$ s$^{-1}$; $k_{app}$ for the NEM-modified enzyme was $5.4 \times 10^{-4}$ s$^{-1}$ with a maximum deviation of ±0.4 $\times 10^{-4}$ s$^{-1}$. The variations affect $\Delta k_{max,app}$ and $k_{app}$ but have no effect on the determination of $[L]_{0.5}$ and $n_H$ (see below).

Data Analysis—Inactivation protection data were analyzed by plots of $\Delta k$ versus [L] and 1/$\Delta k$ versus 1/[L] where $\Delta k$ is the difference between $k_{app}$ in the absence of the varied ligand, L, and $k_{app}$ in the presence of the varied ligand. $\Delta k$ was obtained at five or more different concentrations of L. $\Delta k_{max,app}$ was obtained by extrapolating the 1/$\Delta k$ versus 1/[L] plot to the vertical axis. The limiting inactivation constant ($k_{lim}$) representing the $k_{app}$ at a saturating concentration of varied ligand can be calculated from $k_{app}$ in the absence of the varied ligand and $\Delta k_{max,app}$. Neither $\Delta k_{max,app}$ nor $k_{lim}$ are true constants but rather, depend on the concentrations of any nonvaried protective ligands present and the particular combination of varied and nonvaried ligands examined. $[L]_{0.5}$, the concentration of varied ligand yielding 0.5 $\Delta k_{max,app}$ (i.e., half-maximal protection) at any nonvaried ligand concentration (including zero) was obtained by extrapolating linear reciprocal plots to the horizontal axis. If the primary plot was sigmoidal, $[L]_{0.5}$ was obtained as the varied ligand concentration required to double the vertical axis intercept of the concave-up reciprocal plot. The Hill coefficient, $n_H$, was obtained from the slope of the log ($\Delta k/ (\Delta k_{max,app} + \Delta k)$) versus log [L] plot.

RESULTS AND CONCLUSIONS

Sulfhydryl Group Modification under Nondenaturing and Denaturing Conditions—Under nondenaturing conditions, one free SH group per subunit of ATP sulfurylase is modified by DTNB. This group is designated SH-1. Upon addition of 0.01% (w/v) SDS, two additional SH groups become accessible to DTNB (Fig. 1A). Higher concentrations of SDS or 2 M guanidine HCl increase the rate of modification but do not alter the end point. Thus, we can conclude that the P. chrysogenum ATP sulfurylase contains three SH groups per subunit, two of which (SH-2 and SH-3) are buried and inaccessible to DTNB under nondenaturing conditions. The third SH group can be further differentiated with pCMB. Under nondenaturing conditions, pCMB almost instantaneously modifies SH-1 and then reacts more slowly with SH-2. The third SH group becomes accessible only after the addition of 0.01% SDS (Fig. 1B).

In other experiments, the enzyme was modified with NEM or A-485 in the absence of SDS. After removal of the unreacted reagent by repeated washings through a membrane filter, the modified enzyme was treated with DTNB. No reaction occurred until 0.01% SDS was added. Then, the formation of thionitrobenzoate equivalent to 2 mol of enzyme sites was observed. Thus, NEM and A-485, like DTNB, modify only SH-1 under nondenaturing conditions. The SH-1-modified enzyme eluted from a TSK-4000 gel filtration column at the same retention time as the native enzyme. Thus, modification had no effect on the quaternary structure.

Relationship between SH-1 Modification and Enzyme Activity—Fig. 2 shows the effect of DTNB treatment under nondenaturing conditions on the residual activity of ATP sulfurylase. The decision as to whether modification of the SH group has a significant effect on enzyme activity and the nature of that effect depends on the postmodification assay conditions. When both substrates in the assay mixture were near saturating, DTNB had very little effect. $V_{max}$ was reduced to about 80% of the control value.) When only one substrate was subsaturating, DTNB appeared to affect a partial inactivation. When both substrates were sufficiently subsaturating, DTNB appeared to act as a classical modifier—inactivating agent (activity → zero as $t → \infty$). The results are consistent with an effect of modification on the $K_m$ value of each substrate with little effect on $V_{max}$. In the absence of DTNB, no loss of activity was observed in any of the substrate concentration sets.

The semilog plot for "inactivation" or modification by DTNB appeared to be linear when the half-life ($t_{1/2}$) was about 2 min or less. If the reaction rate was decreased by decreasing the DTNB concentration and/or omitting Mg$^{2+}$, the semilog plots displayed a lag. The cause of the lag is unknown. One possible explanation (other than experimental error) is that subunit modification is partially cooperative. Whatever the cause, it is obvious from Fig. 3 that the loss of enzyme activity paralleled modification of SH-1. The result suggests that modification of a subunit causes a decrease in activity of only that subunit. However, we cannot exclude the alternative explanation that modification of any one subunit causes a partial but equal loss of activity in all associated subunits.

Another explanation that was considered is that DTNB binds slowly to the enzyme prior to modification of SH-1. However, when the initial slopes of the semilog plots were used to calculate $k_{app}$, the replott of $1/k_{app}$ versus 1/[DTNB] was linear extrapolating to the origin. Thus, there is no evidence for the formation of an E·DTNB complex.
Protection against SH-1 Modification by Reversibly Bound Ligands—SH-1 is not required for catalysis and is not essential for substrate binding. Yet a reciprocal relationship exists between SH-1 modification and MgATP binding. Modification appears to have increased the [S]o for MgATP (verified of DTNB. Ligands-SH-1 is not required for catalysis and is not essential for substrate binding. Yet a reciprocal relationship exists between SH-1 modification and MgATP binding. Modification appears to have increased the [S]o for MgATP (verified in a later section) and, as shown in Fig. 4, MgATP binding protected the SH group against modification by DTNB. Note that in the presence of high MgATP, the lag in the semi-log modification plot is quite obvious. As a check on the above results, enzyme activity after different modification times was measured at low substrate concentrations. (The carryover of MgATP into the assay medium was negligible.) The plots were quite similar to those shown in Fig. 4.

SO42- at 10 mM (~12 Keq) had only a small effect on the rate of modification or enzyme inactivation by DTNB at pH 8.0 either in the absence or presence of 5 mM Mg2+(kapp was reduced to 87% of the control value), but APS, like MgATP, protected against both processes. At 8 μM (saturating) APS, the semi-log plot appeared to be linear; kapp was about 4% of the control value. In the absence of other ligands, 5 mM Mg2+ nearly doubled kapp for inactivation by DTNB.

Initial Velocity Kinetics of the Native and SH-modified Enzyme—In order to characterize the effect of SH-1 modification more completely, the kinetics of the native and fully SH-1 modified enzyme were studied. Native ATP sulfurylase displayed hyperbolic v versus [S] plots and normal (linear) reciprocal plots (Fig. 5A). The kinetic constants of the enzyme at pH 8.0 and 30 °C were: Kma = 0.2 mM, Kmb = 0.8 mM, and Vmax = 9.0 units × mg protein−1 where A = MgATP and B = SO42−. In contrast, the DTNB-treated enzyme displayed sigmoidal v versus [S] plots and nonlinear, concave-up reciprocal plots (Fig. 5B). Modification of the SH group had only a small effect on Vmax, but the [S]0.5 values were increased more than 19-fold and 14-fold to 3.8 mM and 11.5 mM for MgATP and sulfate, respectively. Hill plots (Fig. 5C) were linear with slopes (i.e. Hill coefficients, nH) of 1 for the native enzyme and 2 for the modified enzyme.

The experiment depicted in Fig. 5B was conducted at a near-saturating level of the fixed cosubstrate. At 1 mM MgATP, the Vmax and [S]0.5 for SO42− were reduced to 1.5 units × mg protein−1 and 2.3 mM, respectively, but nH was still 2. At 5 mM SO42−, the Vmax and [S]0.5 for MgATP were reduced to 2.9 units × mg protein−1 and 1.1 mM; nH remained 2.

Treatment of the DTNB-modified enzyme with 10 mM NaCN (after removal of the unreacted DTNB) resulted in the stoichiometric release of thionitrobenzoate (1 mol/mol of subunit; data not shown). The remodified enzyme (presumably cyanlated at each SH-1) displayed the same sigmoidal kinetics as that of the original DTNB-modified form. The enzyme modified with NEM, or tetranitromethane, or A-485 also displayed sigmoidal kinetics with respect to both substrates; nH was 2 in all cases. Thus, the sigmoidal kinetics can be attributed solely to the covalent modification of SH-1. The chemical nature or size of the substituent seems to be unin-
Cooperative Ligand Binding to SH-modified ATP Sulfurylase

FIG. 4. Progress of SH-1 modification by DTNB in the presence of MgATP. The enzyme (4 μM total sites) was incubated with 50 μM DTNB at pH 8 (0.04 M Tris-Cl) and 30 °C in the presence of 5 mM free Mg²⁺ and the indicated concentrations of MgATP.

FIG. 5. Initial velocity kinetics of the native and DTNB-treated ATP sulfurylase. A, v versus [S] for the native enzyme. When [MgATP] was varied, [SO₄⁻] was maintained at 40 mM. When [SO₄⁻] was varied, [MgATP] was maintained at 20 mM. The enzyme concentration in the assay ranged from 0.1 to 20 units × mg protein⁻¹. B, v versus [S] for the SH-modified enzyme. The enzyme was modified by incubating the enzyme (128 μg of protein) with 30 mM DTNB at pH 8.0, 30 °C for 12 min. The solution was then diluted 5-fold in ice-cold 50 mM Na-EPPS buffer, pH 8.0. The assay conditions were the same as those described in A except 50 mM SO₄⁻ was used when MgATP was varied. The enzyme concentration in the assay ranged from 0.25 to 0.5 μg × ml⁻¹. Inset, reciprocal plot of primary data. Vmax is 9 units × mg protein⁻¹. C, Hill plots of data shown in A and B.

Chemical Inactivation Kinetics of the Native and SH-modified Enzymes—Sigmoidal velocity curves and activation by competitive inhibitors are characteristics of positively cooperative ligand binding. However, there are a number of purely kinetic phenomena that can yield the same qualitative patterns (see “Discussion”). Consequently, it was necessary to determine whether MgATP and SO₄⁻ (or suitable analogs) displayed cooperative binding under equilibrium, nonturnover conditions. In lieu of direct binding measurements, which were impractical because of the high [S]₀.₅ values, we attempted to characterize the equilibrium interaction of ligands with the enzyme by analyzing the protection provided by a ligand against chemical inactivation by phenylglyoxal (an arginine-targeted reagent). The major objective was to determine whether the ligand concentration dependence of protection was hyperbolic or sigmoidal (see Appendix 1). Preliminary experiments showed that phenylglyoxal did not attack SH-1 (3) nor release thionitrobenzoate from enzyme previously modified with DTNB. However, to insure stability of the modification, these experiments were conducted with the NEM-modified enzyme. Residual enzyme activity was measured at 15 mM MgATP and 30 mM SO₄⁻. Thus, a decrease in

The same sigmoidal behavior was seen for both substrates when MoO₄⁻ was used in place of SO₄⁻. Because the molybdate assay couples the ATP sulfurylase reaction to adenylate kinase (myokinase) instead of APS kinase (8), the sigmoidicity cannot be a consequence of an altered interaction between the two sulfate activating enzymes.

Activation of the SH-1 Modified Enzyme by Fluorosulfonate and Other Sulfate Analogues—Fluorosulfonate (FSO⁻) is a potent inhibitor of ATP sulfurylase, competitive with SO₄⁻ and apparently uncompetitive with MgATP (8). At near-saturating MgATP and unsaturating SO₄⁻, the v versus [FSO⁻] plot for the native enzyme was hyperbolic descending. At 15 mM MgATP and an ~[S]₀.₅ level of SO₄⁻, the DTNB-modified enzyme was activated by low concentrations of FSO⁻ (Fig. 6). The same effect was observed with ClO₄⁻, NO₃⁻, FPO₄⁻, and S₂O₃²⁻ (Table I). Moreover, the relative analog concentrations required for maximum activation were in the same order as the limiting K values of the compounds as dead-end inhibitors of the unmodified enzyme (e.g. the K values for FSO⁻ and S₂O₃²⁻ are 3.4 and 360 μM, respectively) (8).

In the presence of 25 mM MgATP, 5 mM excess Mg²⁺, and 350 μM FSO⁻, the v versus [SO₄⁻] plot for the modified enzyme was hyperbolic (n_H = 1). The K_MSP for SO₄⁻ was increased to about 50 mM, but Vmax remained about 7 units × mg protein⁻¹. Thus, FSO⁻ behaved as a dead-end inhibitor that competes with and mimics a substrate that binds cooperatively (p. 387 of Ref. 28).

Chemical Inactivation Kinetics of the Native and SH-modified Enzymes—Sigmoidal velocity curves and activation by competitive inhibitors are characteristics of positively cooperative ligand binding. However, there are a number of purely kinetic phenomena that can yield the same qualitative patterns (see "Discussion"). Consequently, it was necessary to determine whether MgATP and SO₄⁻ (or suitable analogs) displayed cooperative binding under equilibrium, nonturnover conditions. In lieu of direct binding measurements, which were impractical because of the high [S]₀.₅ values, we attempted to characterize the equilibrium interaction of ligands with the enzyme by analyzing the protection provided by a ligand against chemical inactivation by phenylglyoxal (an arginine-targeted reagent). The major objective was to determine whether the ligand concentration dependence of protection was hyperbolic or sigmoidal (see Appendix 1). Preliminary experiments showed that phenylglyoxal did not attack SH-1 (3) nor release thionitrobenzoate from enzyme previously modified with DTNB. However, to insure stability of the modification, these experiments were conducted with the NEM-modified enzyme. Residual enzyme activity was measured at 15 mM MgATP and 30 mM SO₄⁻. Thus, a decrease in
Activation of DTNB-modified ATP sulfurylase by native and DTNB-modified ATP sulfurylase.

The native enzyme was assayed at 10 mM MgATP, 5 mM excess Mg**, and 0.5 mM SO**-.

The modified enzyme was assayed at 14.4 mM MgATP, 5 mM excess Mg**, and 5 mM SO**-.

For the enzyme preparations used in this experiment, the above substrate concentrations provided uninhibited velocities of 0.27 Vmax and 0.21 Vmax, respectively.

**TABLE I**

Activation of DTNB-modified ATP sulfurylase by sulfate analogs

Activity was measured at 15 mM MgATP, 5 mM Na2SO4, 5 mM excess MgCl2, and varying concentrations of analog at pH 8.0, 30 °C.

The control rates (taken as 100) were 1.20-1.5 units.

| Compound added | Concentration required for maximum effect | Relative maximum activity |
|----------------|------------------------------------------|--------------------------|
| None           |                                         |                          |
| KFSO4          | 35                                       | 100                      |
| KClO4          | 30                                       | 205                      |
| KCl            | 150                                      | 205                      |
| NaNO2          | 300                                      | 191                      |
| Na2SO4         | 2000                                     | 184                      |
| NaFPO4         | 5000                                     | 249                      |

The enzyme activity reflected true inactivation. The maximum carryover of phenylglyoxal (60 μM) had no effect on the assay. The semi-log inactivation plots were linear for the native and for the NEM-modified enzyme, both in the absence of added ligands and in the presence of a single ligand, or combinations which did not promote catalytic activity. In most cases, protection by bound ligands was partial. That is, inactivation proceeded at a measurable rate even at a saturating concentration of a protective ligand. FSO4- in the absence of MgATP was the exception. Partial protection was not a hindrance to analysis because the difference between kapp in the absence of the varied protector and kapp at a saturating concentration of that protector (Δkapp) was large enough to analyze by plots of Δk versus [ligand] and the corresponding reciprocal and Hill plots. This procedure has been used successfully to examine the interaction of an enzyme with a single ligand (10, 17–24) and with multiple ligands (12, 25, 26). Some of the results are summarized in Table II and described below.

(a) The SH-modified enzyme was more susceptible than the native enzyme to inactivation by phenylglyoxal. In the absence of ligands, k, the first order rate constant for inactivation of the native enzyme was 2.67 ± 0.25 × 10⁻⁶ s⁻¹ while k for the SH-modified enzyme was double at 5.44 ± 0.27 × 10⁻⁶ s⁻¹ (means ± standard deviations for 10 and 11 measurements, respectively). For any single protective ligand (first five rows of Table II), the Δkapp,exp for the SH-modified enzyme was 1.9 ± 0.3 times that for the native enzyme (mean ± maximum range). Thus, the increased susceptibility of the SH-modified enzyme to phenylglyoxal resulted mainly from an increase in the reactivity of one or more ligand-protectable groups.

(b) None of the Δk versus [ligand] plots for the native enzyme displayed an inflection. Log-log Hill plots of Δk/ (Δkmax,exp − Δk) versus [L] were linear with slopes of 0.98 ± 0.06 (mean ± standard deviation). In the absence of a coligand, the plots of Δk versus [varied ligand] for the SH-modified enzyme had no inflection; nH values were close to unity (1.00 ± 0.02 S.D.). However, in the presence of MgATP, the plots of Δk versus [FSO4-] or [S2O8^-2] were sigmoidal (e.g. Fig. 7). Similarly, in the presence of FSO4- or S2O8^-2, the plot of Δk versus [MgATP] was sigmoidal. The nH values were all close to 2 (1.92 ± 0.09 S.D.). It appears that under equilibrium conditions, both FSO4- and MgATP bind cooperatively to the SH-modified enzyme if a cosubsite ligand is present. In other words, cooperativity is evident only in the formation of a ternary complex.

(c) The [L]0.5 for MgATP in the absence of a coligand was 0.19 mM for the native enzyme, in reasonable agreement with the K0.5 of 0.25–0.42 mM determined from initial velocity studies (3, 8, 9). Added S2O8^-2 did not alter the [L]0.5 for MgATP; in agreement with the noncompetitive inhibition by S2O8^-2 with respect to MgATP (8). [MgATP]0.5 for the SH-modified enzyme was 1.5 mM.

(d) The [L]0.5 for SO4^-2, S2O8^-2, or FSO4- in the absence of MgATP did not increase after SH modification. (The values actually decreased.) The [L]0.5 of the native enzyme for SO4^-2 in the absence of MgATP was 5.7 mM. This value is higher than K0.5 calculated from various forward reaction initial velocity data (0.6–1.7 mM) but close to the K0.5 obtained when SO4^-2 was used as a product inhibitor of the reverse reaction (4.4 mM) (9).

(e) The [L]0.5 for FSO4- at 15 mM (near saturating) MgATP was 1.7 μM, which is in the same range as the K0.5 of 3.4 μM obtained from steady state kinetics studies (8). The corresponding value for the SH-modified enzyme was 33 μM, in accordance with the increased [S2O8^-2] for the normal subsite ligand (SO4^-2) at high [MgATP]. In the absence of MgATP, the respective [FSO4-]0.5 values were 30 μM and 11.6 mM confirming earlier conclusions (8) that FSO4- has a much greater affinity for E-MgATP than for free E.

(f) At 0.19 mM MgATP, the [L]0.5 for S2O8^-2 of the native enzyme was 0.13 mM, which is in reasonable agreement with the K0.5 of 0.35 mM obtained from steady state inhibition studies under nearly identical conditions (8).

(g) Free ATP ([L]0.5 = 9 μM) binds more tightly than MgATP ([L]0.5 = 0.19 mM) to the free native enzyme. However, FSO4-, which is an effective protector in the presence of 0.19 mM MgATP, ([FSO4-]0.5 = 11 μM) did not provide additional protection even at 10 mM in the presence of 9 μM free ATP. The result suggests that FSO4- does not bind with any significant affinity to E-ATP and is consistent with our earlier observation that FSO4- would promote [³²P]ATP binding in the presence but not in the absence of Mg** (9). (Binding is either compulsory ordered with MgATP before ATP.)

**DISCUSSION**

Native ATP sulfurylase from *P. chrysogenum*, an oligomeric enzyme, exhibits normal hyperbolic velocity curves for both substrates (MgATP and SO4^-2 or MoO4^-2). However, chemical modification of a single SH group per subunit causes the v
versus [S] plots for both substrates to become sigmoidal with $n_1$ values of 2. There are two basic causes of sigmoidal velocity curves. The first is positive cooperativity in substrate binding, an equilibrium process that (theoretically) can be observed in the absence of substrate turnover. Equilibrium cooperativity requires the interaction of two or more subunits (or sites) and can be described in terms of the concerted symmetry model (27, p. 421 of Ref. 28), the sequential interaction model (29, p. 355 of Ref. 28), or, if applicable, the oligomer dissociation model (30, 31, p. 457 of Ref. 28). A second cause of sigmoidal velocity curves is the "V-type" substrate activation system in which the catalytic activity of occupied sites increases progressively as vacant sites are filled (p. 357 of Ref. 28). Further, the Hill plot was clearly linear over the range 0.1-0.9 $V_{max}$. The experimental velocity curves (Fig. 5) have inflection points at about 0.25 $V_{max}$ and the Hill plots were linearly over the range 0.1-0.9 $V_{max}$. The $n_1$ values were close to 2.

MgATP and SO$_4^{2-}$ bind randomly to native ATP sulfurylase (9). Thus, it is easy to imagine that SH modification could significantly alter certain rate constants and set up the conditions for two kinetically unequal paths to the central complex. However, this model was unacceptable for three reasons. First, the steady state random AB mechanism provides for sigmoidal behavior only for the substrate initiating the faster route. Second, the mechanism predicts partial substrate inhibition when the substrate initiating the slower path is varied. Both MgATP and SO$_4^{2-}$ produced sigmoidal velocity curves at > [S], of the fixed cosubstrate (Fig. 5) and in neither plot was substrate inhibition observed. Third, the random mechanism does not provide for activation by a dead-end competitive inhibitor.

The substrate-dependent product release mechanism assumes that product release from an abortive ternary E- product-substrate complex is faster than release from the normal ternary E- MgATP. A compulsory alternating site mechanism of this type produces normal hyperbolic velocity

| Varied ligand | Native enzyme $\Delta V_{max}$ ($[L]_{0.5}$) | $n_1$ | NEM-modified enzyme $\Delta V_{max}$ ($[L]_{0.5}$) | $n_1$ |
|--------------|----------------------------------|------|----------------------------------|------|
| MgATP | 0.19 mM | 1.0 | 0.19 mM | 1.0 |
| MgATP | 100 mM | 2.0 | 0.19 mM | 1.0 |
| MgATP | 2.5 mM SO$_4^{2-}$ | 3.0 | 0.19 mM | 1.0 |
| SO$_4^{2-}$ at an [L]$_{0.5}$ of MgATP | 1.5 | 0.13 mM | 1.0 | 2.7 | 0.81 mM | 1.8 |
| MgATP at 15 $\mu$M FSO$_4^{-}$ | 2.0 | 50 $\mu$M | 0.9 | 5.2 | 0.12 mM | 2.0 |
| MgATP at 100 $\mu$M FSO$_4^{-}$ | 1.9 | 0.19 mM | 1.0 | 2.9 | 1.2 mM | 1.9 |
| SO$_4^{2-}$ at an [L]$_{0.5}$ of MgATP | 2.1 | 5.6 mM | 1.0 | 3.3 | 2.5 mM | 1.0 |
| SO$_4^{2-}$ at an [L]$_{0.5}$ of MgATP | 1.0 | 0.19 mM | 1.0 | 3.8 | 1.5 $\mu$M | 1.0 |
| MgATP at 15 $\mu$M FSO$_4^{-}$ | 0.5 | 1.7 $\mu$M | 1.0 | 1.9 | 33 $\mu$M | 1.9 |
| MgATP at 9 $\mu$M ATP (-Mg$^{2+}$ + 0.5 mM EDTA) | 0.9 | 6 $\mu$M | 1.0 | 3.5 | 32 $\mu$M | 2.0 |
| MgATP at 100 $\mu$M FSO$_4^{-}$ | 1.9 | 0.19 mM | 1.0 | 2.9 | 1.2 mM | 1.9 |

a The concentration of enzyme sites for the experiment with the native enzyme was reduced to 0.09 $\mu$M to minimize depletion of added FSO$_4^{-}$ upon formation of E $\cdot$ MgATP $\cdot$ FSO$_4^{-}$.

b MgATP was 0.19 mM with the native enzyme and 1.5 mM with the modified enzyme.

c Low MgATP was 0.34 mM with the native enzyme, and 0.68 mM with the NEM-modified enzyme. Fig. 7 shows the plotted results for this experiment.
Cooperative Ligand Binding to SH-modified ATP-Sulfurylase

FIG. 7. Inactivation of native and NEM-modified ATP sulfurylase by phenylglyoxal. The native and NEM-modified enzymes (~0.5 μM sites), were incubated with 3 mM phenylglyoxal at pH 8.0 (0.05 M Na-EPPS buffer) and 30 °C in the presence of 0.34 mM MgATP (native enzyme) or 0.68 mM MgATP (modified enzyme) and varied [FSO₃⁻]. The incubation volumes were 0.22 ml. A and B, semi-log plots of remaining activity for the native and SH-modified enzyme, respectively, assayed at 15 mM MgATP and 30 mM SO₃⁻. C and D, plots of Δk versus [FSO₃⁻] for the native and SH-modified enzyme respectively, where Δk = (k in the presence of the fixed [MgATP] and zero FSO₃⁻) − (k at the fixed MgATP and indicated FSO₃⁻ concentrations). Insets, plot of 1/Δk versus 1/[FSO₃⁻]. E, Hill plots.

Γ<sub>Γ</sub>
curves. But if SH modification partially uncouples the interacting subunits, two kinetically distinct paths of APS release would be generated, slow and site-independent; at low substrate levels changing to faster and alternating site at higher substrate levels. This mechanism can also accommodate activation by nonreactive substrate analogs. Thus, this model cannot be disregarded on the basis of the experimental kinetic data.

In its simplest form, the mnemonic enzyme mechanism assumes that the free enzyme can exist in two slowly interconverting forms: $E_1$ (with a low substrate affinity) and $E_2$ (with a high substrate affinity) of which only $E_1$ is regenerated at the conclusion of a catalytic cycle. Addition of substrate $A$ to either form yields the same $E_{A}B$ species. Sigmoidicity results from a shift in the flux from $E_1 \rightarrow E_{1}A \rightarrow E_{1}AB \rightarrow E_1 +$ products to $E_{2}A \rightarrow E_{2}AB \rightarrow E_{2} +$ products as $[A]$ is increased. Low concentrations of a dead-end inhibitor, $I$, could activate the reaction at low substrate if $I$, like $A$, induces the $E_2$ conformation which then persists long enough to bind $A$ after $I$ dissociates. On the basis of kinetics alone, the mnemonic enzyme mechanism was a viable possibility for SH-modified ATP sulfurylase. The same intermediates could be involved in the reaction catalyzed by the native enzyme, but $E_1$ and $E_2$ would have to be in rapid equilibrium to yield hyperbolic velocity curves. In other words, we would have to postulate that the effect of SH modification is to slow the interconversion of the two free enzyme species.

Because two kinetically based mechanisms yielding sigmoidal velocity curves could not be eliminated, we determined the equilibrium binding curves for MgATP, $S_{2}O_{3}^{2-}$, and $F_{SO_{3}}^{2-}$ (the latter two are nonreactive sulfate analogs). The binding curves were established from the protection provided by a varied ligand against phenylglyoxal-dependent inactivation. Both the native and the SH-modified enzyme displayed hyperbolic plots of $k$ versus $[ligand]$ in the absence of a coligand. For the native enzyme, the $k$ plots also were hyperbolic in the presence of a fixed concentration of coligand. But the SH-modified enzyme displayed sigmoidal plots of $k$ versus $[FSO_{3}^{2-}]$ or $[S_{2}O_{3}^{2-}]$ in the presence of MgATP and sigmoidal plots of $k$ versus $[MgATP]$ in the presence of $F_{SO_{3}}^{2-}$ or $S_{2}O_{3}^{2-}$. The $n_H$ values were 1.8-2.0. The sigmoidicity is not a result of synergetic protection by the two ligands. It can be shown that if MgATP and a sulfate analog bind noncooperatively to their respective subunits, the plots of $k$ versus [ligand] will be hyperbolic as long as only one ligand is varied at a constant concentration of the coligand (Appendix 1). Even if the binding of one molecule of ligand protects multiple subunits (42), the $k$ versus [ligand] plots will not be sigmoidal (Appendix 2). The cumulative results support positively cooperative ligand binding as the cause of the sigmoidal velocity curves. However the cooperativity is evident only in the formation of the ternary complex, i.e.: both $F_{SO_{3}}^{2-}$ or $S_{2}O_{3}^{2-}$ and MgATP bind hyperbolically to the free SH-modified enzyme, but cooperatively to the appropriate binary complex. The $n_H$ of 2 could reflect weak cooperativity between all subunits of the oligomer or strong cooperativity restricted to interacting pairs of subunits.

Several important questions remain to be answered including: (a) is there any regulatory significance to the sigmoidal response of the SH-modified enzyme? Or, to phrase it differently: Does SH modification occur in vivo and if so, what is the modifying agent? Clearly, a reversible modification of SH-1 in vivo would modulate enzyme activity over a wide range at the normal cellular level of ATP. One can easily envision a process whereby the availability of NADPH controls the native/modified ATP sulfurylase ratio through the interme-

diacy of some sulfhydryl/disulfide agent, e.g. glutathione. (NADPH is needed at a 4:1 ratio for each molecule of $SO_{3}^{2-}$ reduced to H$_2$S for cysteine biosynthesis). A decrease in the cellular level of NADPH would result in an increase in the level of oxidized glutathione (GSSG) which might "turn off" ATP sulfurylase by GSSG-enzyme SH interchange. Another possibility is that SH modification is a negative feedback process that reduces the activity of the $SO_{3}^{2-}$ assimilation pathway when the cells have sufficient reserves of reduced organic sulfur. Glutathione and $S$-adenosylmethionine are potential effectors for this process. (The latter might serve as an SH-methylating agent.) SH modification may also be a mechanism for deactivating ATP sulfurylase when the cellular ATP falls below the apparently essential level of ~1 mM (43). It is also possible that in vitro SH modification induces the same conformational change in the enzyme that is normally triggered in vivo by a reversibly bound effector or perhaps by phosphorylation or adenylation (etc.). Because ATP sulfurylase is the first enzyme in a branched biosynthetic pathway, these possibilities are worthy of further consideration.

If SH modification has no physiological role and does not occur in vivo, then a different question becomes important: (b) is there any relationship between the sigmoidal response of the SH-modified enzyme and the mechanism of the native enzyme? To put it another way, does the mechanism of the native enzyme involve subunit interactions which are kinetically invisible (i.e. $n_H = 1$) but which become evident when the enzyme is forced into an unnatural state?

Finally, it has also occurred to us that the sigmoidal response may be an accident of SH modification with no significance to either metabolic regulation or the mechanism of the native enzyme. But even in this case, the phenomenon is relevant to considerations of protein evolution because it demonstrates how easy it is to convert a hyperbolically responding enzyme to one that displays positive binding cooperativity.

APPENDIX

1) Kinetics of Enzyme Inactivation in the Presence of Two Partially Protective Ligands that Form a Catalytically Inactive Ternary Complex

Scheme 1 shows the general model for chemical inactivation of a noncooperative enzyme by reagent I in the presence of two reversibly bound ligands that add randomly to form a catalytically inactive ternary complex. Thus, for ATP sulfurylase, $A$ could be MgATP while $X$ would be $F_{SO_{3}}^{2-}$. Alternatively, if $A$ is a nonreactive MgATP analog, $X$ could be $SO_{3}^{2-}$ or $MoO_{4}^{2-}$. $K_A$ and $K_X$ are the ligand dissociation constants of $A$ and $X$ from their respective binary complexes. The symbols $\alpha K_A$ and $\alpha K_X$ are the respective ligand dissociation constants of the ternary complexes. In the presence of a large excess of

\[ \begin{align*}
E^{(inactive)} & + I \rightarrow E^{(inactive)}I \\
E^{(inactive)}I + X & \rightarrow E^{(inactive)}IX \\
E^{(inactive)}I + A & \rightarrow E^{(inactive)}IA \\
E^{(inactive)}IA + X & \rightarrow E^{(inactive)}IAX \\
E^{(inactive)}IA + X & \rightarrow E^{(inactive)}IAX.
\end{align*} \]
Cooperative Ligand Binding to SH-modified ATP-Sulfurylase

16287

reagent I, the modification of free E proceeds with a pseudo first order rate constant $k_1[1]$, which can be designated simply as $k$. The reactions of I with the ligand-occupied species are expressed in terms of $k$, e.g. $EA$ is modified with a rate constant $k, [1]$ indicated as $k, I$. The model makes no assumption about the number or location of I-sensitive groups present, only that modification of any one of them is sufficient to inactivate an active site. For example, there could be a single I target group. Ligand A, when bound to the enzyme reduces the rate of modification but does not prevent the reaction. Similarly, bound X alone partially protects against modification, while A and X together protect to a greater extent but not completely. There could also be (e.g.) three different I target groups essential for activity, one of which can be completely or partially shielded by bound A, another by bound X, while the third is equally accessible to I in all enzyme species. A bound ligand may wholly or partially protect one target group while inducing a conformational change in the enzyme that increases the reactivity of another target group. The different possibilities only alter the physical meanings of $k, I, \beta k, \gamma k, \delta k$, etc. but have no effect on the first equation. Scheme 1 assumes that if the enzyme is oligomeric, modification of one subunit has no effect on the rate of modification of another subunit. It is also assumed that the rate of inactivation is much slower than the rate of ligand dissociation so that the various active enzyme species remain at equilibrium throughout the modification period.

The rate of inactivation is given by:

$$-d[E]_0 \over dt = k[E] + \beta k[EA] + \gamma k[EAX] + \delta k[EAX]$$

where $[E]_0$ represents total active site (subunit) concentration (proportional to measured activity). Substituting for $[E]$, $[EA], \text{etc.}$ in terms of $[E]_0$, rearranging and integrating, we obtain the usual first order equation for the loss of enzyme activity:

$$\ln \left( \frac{[E]}{[E]_0} \right) = -k_\text{app} t$$

where $k_{\text{app}}$, the apparent first order rate constant at any fixed $[A]$ and $[X]$ is given by:

$$k_{\text{app}} = \frac{\left( 1 + \frac{[A]}{K_A} + \frac{[X]}{K_X} + \frac{[A][X]}{K_A K_X} \right)^{\alpha K_A K_X}}{\left( 1 + \frac{[A]}{K_A} + \frac{[X]}{K_X} + \frac{[A][X]}{K_A K_X} \right)^{\alpha K_A K_X}}$$

The symbols $[A]$ and $[X]$ refer to the concentrations of free ligand at equilibrium with the enzyme sites.

At any fixed $[A]$, a plot of $\Delta k$ versus $[X]$ is hyperbolic yielding linear reciprocal plots:

$$\frac{1}{\Delta k} = \frac{k_{\text{app}}}{\Delta k_{\text{max,app}}} \frac{1}{[X]} + \frac{1}{\Delta k_{\text{max,app}}}$$

where:

$$\Delta k = (k_{\text{app}} \text{ at the fixed } [A] \text{ alone}) - (k_{\text{app}} \text{ at the same } [A] \text{ plus the experimental } [X]), \text{ e.g. } \Delta k \text{ is a measure of the protection provided by the varied ligand.}$$

$$\Delta k_{\text{max,app}} = (k_{\text{app}} \text{ at the fixed } [A]) - (k_{\text{app}} \text{ at the same } [A] \text{ plus saturating } [X]). \Delta k_{\text{max}} \text{ is a measure of the maximum protection provided by the varied ligand.}$$

$\Delta k_{\text{max,app}}$ for the random system is a complicated function of $k, [A], K_A, \alpha, \beta, \gamma, \text{ and } \delta$. In the absence of $A$, $\Delta k_{\text{max,app}}$ would be equal to $k(1 - \gamma)$. At saturating $A$, $\Delta k_{\text{max,app}} = k(\beta - \delta)$. The limiting $\Delta k_{\text{max}}$ (the difference between $k_{\text{app}}$ in the absence of either ligand and $k_{\text{app}}$ at saturating $[A]$ and $[X]$) is $k(1 - \delta)$. A successful analysis requires that $\Delta k_{\text{max,app}}$ be large enough to provide measurable $\Delta k$ values as the ligand concentration is varied. $k_{\text{app}}$ is given by:

$$k_{\text{app}} = \frac{k_A \left( 1 + [A] \right)}{1 + \frac{[A]}{K_A}}$$

or

$$\frac{-d[E]_0}{dt} = k_\text{app}$$

where $K_A \text{ is the total concentration of subunits (proportional to enzyme activity) and } [E]_0 \text{ is the total concentration of dimer.}$ If $K_A$ is the intrinsic $A$ dissociation constant, the rate of activity loss in the presence of $A$ is given by:

$$\frac{-d[E]_0}{dt} = k_\text{app}$$

where $K_A$ is the total concentration of subunits (proportional to enzyme activity) and $[E]_0$ is the total concentration of dimer. If $K_A$ is the intrinsic $A$ dissociation constant, the rate of activity loss in the presence of $A$ is given by:

$$\frac{-d[E]_0}{dt} = k_\text{app}$$

or

$$\Delta k_{\text{max,app}}$$
Equation (10) can be integrated to the usual form:

\[
\ln \left( \frac{[E]}{[E]_0} \right) = -k_m t \quad \text{where:} \quad k_m = \frac{k}{\left(1 + \frac{[A]}{K_A}\right)^n} \tag{11}
\]

Thus the semilog plot at any [A] is linear. \( \Delta k \) can be defined as \( k - k_{app} \). The effect of varied [A] can then be written as

\[
\Delta k = \frac{2[A] + [A]\left(1 + \frac{[A]}{K_A}\right)^n}{K_A\left(1 + \frac{[A]}{K_A}\right)^n} \tag{12}
\]

If the limiting \( k_{app} \) at [A] = \infty is zero, \( \Delta k_{max} \) is equal to \( k \) and equation (12) reduces to:

\[
\frac{1}{\Delta k_{max}} = \frac{1}{K_A} + \frac{1}{[A]} + \frac{1}{\Delta k_{max}} \tag{13}
\]

or

\[
\frac{1}{\Delta k_{max}} = \frac{1}{K_A}\left(1 + \frac{[A]}{K_A}\right) - 1 \tag{14}
\]

In general, if \( n \) subunits are completely protected by the binding of a single molecule of A, the equation is:

\[
\Delta k_{max} = \frac{1}{K_A}\left(1 + \frac{[A]}{K_A}\right)^n - 1 \tag{15}
\]

A plot of \( \Delta k \) versus [A] will rise more steeply than normal but will not be sigmoidal. Half-maximal protection will occur at an [A]_{0.5} that is less than \( K_A \). The reciprocal plot is nonlinear, decreasing in slope as \( 1/[A] \) decreases and intersecting the vertical axis at \( 1/\Delta k_{max} \). As \( 1/[A] \) increases, the plot approaches a linear asymptote with a slope of \( K_A/n\Delta k_{max} \). The Hill plot will be nonlinear (concave up) with an average slope \( >1 \) at the point corresponding to [A]_{0.9} or between the points corresponding to [A]_{0.9} and [A]_{1.1}. However, even for a hexamer, this slope will be <1.4. If a nonprotectable target group is present, or if the binding of A to one subunit only partially protects the single target group on all subunits, \( \Delta k_{max} \) will equal \( k(1 - \beta) \) (where \( \beta k \) is the limiting rate constant). The general properties of the plots will be the same as those described above.

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\footnote{The fact that most of the [E]_0 values for native ATP sulfurylase (Table II) are somewhat lower than the analogous kinetic K_c values has not escaped notice. But whether the differences are statistically significant and mechanism based, or simply a result of different buffers having been used remains to be determined.}