Random-Order Ternary Complex Reaction Mechanism of Serine Acetyltransferase from *Escherichia coli*

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ABSTRACT: Although serine acetyltransferase (SAT) from *Escherichia coli* is homologous with a number of bacterial enzymes that catalyze *O*-acetyl transfer by a sequential (ternary complex) mechanism, it has been suggested, from experiments with the nearly identical enzyme from *Salmonella typhimurium*, that the reaction could proceed via an acetyl-enzyme intermediate. To resolve the matter, the *E. coli* gene for SAT was overexpressed and the enzyme purified 13-fold to homogeneity. The results of a steady-state kinetic analysis of the forward reaction are diagnostic for a ternary complex mechanism, and the response of SAT to dead-end inhibitors indicates a random order for the addition of substrates. The linearity of primary double-reciprocal plots, in the presence and absence of dead-end inhibitors, argues that interconversion of ternary complexes is not significantly faster than k_{cat}, whereas substrate inhibition by serine suggests that breakdown of the SAT·CoA binary complex is rate-determining. The results of equilibrium isotope exchange experiments, for both half-reactions, rule out a "ping-pong" mechanism involving an acetyl-enzyme intermediate, and a pre-steady-state kinetic analysis of the turnover of AcCoA supports such a conclusion. Kinetic data for the reverse reaction (acetylation of CoA by *O*-acetylserine) are also consistent with a steady-state random-order mechanism, wherein both the breakdown of the SAT· serine complex and the interconversion of ternary complexes are partially rate-determining.

Serine acetyltransferase (SAT;[1] EC 2.3.1.30) catalyzes the first reaction (eq 1) in a two-step process of sulfur assimilation by microorganisms (*1*−*3*) and higher plants (*4*, *5*). Cysteine synthesis, the second step (eq 2), is catalyzed by *O*-acetylserine (thiol)-lyase (EC 4.2.99.8).

$$\text{serine} + \text{AcCoA} \rightarrow \textit{O}\text{-acetylserine} + \text{CoA} \quad (1)$$

$$\textit{O}\text{-acetylserine} + \text{sulfide} \rightarrow \text{cysteine} + \text{acetate} \quad (2)$$

In both *Salmonella typhimurium* and *Escherichia coli* it has been observed that SAT is associated reversibly with approximately 5% of the total cellular *O*-acetylserine (thiol)-lyase, thereby forming a multienzyme complex referred to as cysteine synthase (*2*). Moreover, the flow of substrates through the pathway is regulated by the negative feedback inhibition of SAT by cysteine (*2*). Variants of SAT from both *S. typhimurium* and *Spinacia oleracea* have been reported to follow a double-displacement (ping-pong) mechanism (*6*, *7*). In each case the enzyme was purified from its bacterial or plant source and shown to be free of *O*-acetylserine (thiol)-lyase activity.

The *cysE* gene of *E. coli*, encoding SAT, was cloned and sequenced by Denk and Böck (*8*), and the purification of SAT, free from *O*-acetylserine (thiol)-lyase, was simplified by overexpression in *E. coli* (*9*). Sequence data presented by Downie (*10*) showed that SAT is a member of a family of microbial *O*-acetyltransferases that include the *lacA* gene product of *E. coli*, galactoside acetyltransferase (*11*), and the *nodL* encoded protein of *Rhizobia*, an oligosaccharide acetyltransferase (*12*). Later, Murray and Shaw (*13*) revisited the subject, pointing out that the above well-conserved triad is in fact a subset of proteins from a much larger family of bacterial *O*-acyltransferases, within which, where amino acid sequence, tertiary structures, and mechanisms are known, there is a strong sense of relatedness. The first of such proteins to be studied by X-ray crystallography, the *lpxA* gene product, was shown to be a trimeric protein with a left-handed parallel β-helix fold (*14*). A structurally related microbial acetyltransferase, with specificity for chloramphenicol, has a similar structure (*15*). Moreover, such a folding pattern arises from a hexapeptide repeat, which occurs, albeit to varying degrees, in other members of the larger acyltransferase family which includes SAT. Since the *lacA* (*11*) and *nodL* (*16*, *17*) products are also trimeric and function via a ternary complex (sequential) mechanism, it

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[1] Abbreviations: A, acetyl acceptor substrate; B, acetyl donor substrate; P, acetyl donor product; Q, acetyl acceptor product; CoA, coenzyme A; AcCoA, acetyl coenzyme A; EtCoA, ethyl S-coenzyme A; SAT, serine acetyltransferase; TSE buffer, 50 mM Tris-HCl, pH 7.5, containing NaCl (0.1 M) and EDTA (0.1 mM); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); K_s, kinetically determined binary complex dissociation constant; K_m, kinetically determined ternary complex dissociation constant; K_d, directly determined binary complex dissociation constant; $K_{iA'}$, binary complex inhibitor dissociation constant for the substrate analogue of A; $K_{iA'(B)}$, ternary complex inhibitor dissociation constant for the substrate analogue of A.

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seemed likely that SAT of *E. coli* would prove to have not only a similar tertiary structure but also a kinetic mechanism in common with the other members of the extended family of microbial *O*-acetyltransferases. The present study was undertaken to clarify kinetic issues, since earlier data of Leu and Cook (*6*) were interpreted as evidence for a double-displacement (ping-pong) mechanism involving an acetyl-enzyme intermediate. The results of the kinetic studies presented here argue strongly that SAT of *E. coli*, like each of the microbial *O*-acetyltransferases studied thus far, is likely to have not only the same quaternary structure (a trimer of identical subunits, composed of left-handed parallel β-helices) but also a common kinetic mechanism, one that involves a productive ternary complex of substrates and enzyme, without a covalent enzyme−substrate intermediate.

EXPERIMENTAL PROCEDURES

Chemicals. NAD (grade I) and NADH (grade I) were from Boehringer, and coenzyme A was from Pharmacia. Citrate synthase (porcine heart), malic dehydrogenase (porcine heart), and L-serine-UL-^{14}C were from Sigma. Adenosine 5′-[γ-^{33}P]triphosphate was from Amersham Pharmacia. All other chemicals were reagent grade and were obtained from commercial sources.

Overexpression and Purification of SAT. SAT was overexpressed and purified from the *lon*$^-$ *E. coli* strain E1053 harboring pSAT3 that contains the gene for ampicillin resistance and the *cysE* gene that encodes for SAT (*8*) as described by Hindson and co-workers (*18*).

Preparation of AcCoA and EtCoA. AcCoA was synthesized by the method of Simon and Shemin (*19*) as described by Kleanthous and Shaw (*20*). Ethyl-*S*-CoA was synthesized as described by Lewendon and co-workers (*21*).

SAT Standard Assay. The standard assay mixture contained TSE buffer, 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), AcCoA (0.8 mM), and serine (5 mM) in a volume of 990 μL. The reaction was initiated by the addition of 10 μL of enzyme in TSE buffer containing 1 mM DTNB. One unit of activity is defined as that amount of enzyme catalyzing the formation of 1 μmol of product per minute.

Steady-State Kinetics. All assays were performed in triplicate at 25 °C in a final volume of 1 mL. Unless otherwise stated all steady-state kinetic parameters are the means of at least two independent experiments. Rates were measured continuously with a Uvikon 930 spectrophotometer (Kontron) equipped with an automatic cell changer. Linearity of initial rates was observed in all measurements wherein substrate depletion was judged to be less than 8%. In contrast to the observations of Leu and Cook (*6*), for SAT from *S. typhimurium*, the addition of either glycine or alanine had no effect on the linearity of initial rates.

Forward Reaction Kinetics. The forward reaction was assayed according to Kredich and co-workers (*1*) by monitoring the increase in absorbance at 412 nm due to the reaction of CoA with DTNB. Rates were measured by using an extinction coefficient for 5-thio-2-nitrobenzoate (TNB), at 412 nm, of 1.36×10^4 cm^{-1}·M^{-1}.

Stock solutions of SAT were made up in TSE buffer containing DTNB (1 mM) and were stable over the duration of a typical 1 h kinetic analysis. Kinetic analyses were performed over a matrix of four or five substrate concentrations (for acetyl donor and acceptor) in the range $0.33-5K_m$ unless otherwise stated.

Reverse Reaction Kinetics. Initial rates of acetylation of CoA by *O*-acetylserine were measured by coupling the SAT reverse reaction with citrate synthase (CS) and malate dehydrogenase (MDH) and monitoring the appearance of NADH at 340 nm ($\epsilon^M = 6.22 \times 10^3$ cm^{-1}·M^{-1}), an approach adopted successfully (*20*) for studies of chloramphenicol acetyltransferase (CAT). Each assay mixture contained TSE buffer, pH 7.5, L-malate (5 mM), NAD$^+$ (1.5 mM), NADH (0.11 mM), 2 units of malate dehydrogenase, 4 units of citrate synthase, *O*-acetylserine, CoA, and SAT in a final volume of 1 mL. In a control experiment none of the components of the coupled assay affected the rate of the SAT forward reaction. Under the above conditions, for the coupled assay, 0.93 equivalents of NADH is reported to be produced per equivalent of AcCoA consumed (*22*), and moreover, the stoichiometry is observed only if NADH is present in the starting assay mixture. Stock solutions of SAT (0.25−0.5 μM) were made up in TSE buffer and were marginally unstable with on average between 10% and 15% of the enzyme activity being lost over the duration of a typical 1 h kinetic analysis. Kinetic studies were performed over a matrix of four or five CoA concentrations, in the range $0.33-3K_m$, and four or five *O*-acetylserine concentrations in the range $0.33-2K_m$. Reactions were initiated by the simultaneous addition of SAT and *O*-acetylserine (stock solutions of the latter were prepared for immediate use by dissolving solid *O*-acetylserine hydrochloride in distilled water and adjusting the pH to 6.5 by the dropwise addition of 2 M NaOH).

The kinetic data for both the forward and reverse reactions yielded linear Lineweaver−Burk (*23*) plots over the concentration range of substrates utilized, and therefore the rapid-equilibrium assumption could be applied (*24*, *25*). Kinetic coefficients in the rate equation (eq 3) were derived according to Dalziel (*26*) from linear intercept and slope replots from computer-fitted double-reciprocal plots (*27*):

$$E/v_0 = \phi_o + \phi_A/[A] + \phi_B/[B] + \phi_{AB}/[A][B] \quad (3)$$

where E is the concentration of total enzyme, v_0 is the initial rate of product formation, and each ϕ coefficient is a compound rate constant term for the forward reaction.

K_m and k_1 for the Hydrolysis of AcCoA. Conditions were identical with those of the forward reaction assay except that no serine was incorporated and the concentration of SAT was typically in the range 1−5 μM. All K_m and k_1 values were determined by direct linear plots (*28*).

^{14}C Isotope Exchange at Equilibrium ([^{14}C]Serine to [^{14}C]-O-Acetylserine). An isotope exchange reaction (250 μL) containing SAT (4.8 μM), 1.5 mM [^{14}C]serine (0.91 μCi/μmol), *O*-acetylserine (15 mM), and DTNB (0.1 mM) in TSE buffer and a control acetyltransferase reaction containing SAT (1.9 μM), 0.45 mM [^{14}C]serine (0.91 μCi/μmol), AcCoA (0.2 mM), and DTNB (0.9 mM) in TSE were each incubated at 25 °C. Aliquots (50 μL) were removed from each reaction at 0, 5, 10, 15, and 25 min intervals and quenched by the addition of 10 μL of HCl (1 M). Supernatants were collected by centrifugation and neutralized by the addition of NaOH (1 M), and aliquots were analyzed by ascending chromatography by TLC on a C_{18}-silica gel plate, developed in a solution of 95% methanol containing 5%

aqueous ammonia. Amino acids were visualized by staining with potassium permanganate, and ^{14}C was detected by autoradiography.

Preparation of [^{33}P]CoA. Dephospho-CoA kinase (0.05 unit) was added to a solution (375 μL) containing 4.5 mM [^{33}P]ATP (specific activity 37 mCi/mmol), dephospho-CoA (3.7 mM), $MgCl_2$ (30 mM), and DTT (1 mM) in TSE buffer, and the reaction was incubated for 2 h at 37 °C (*30*). A 5 μL aliquot was diluted 10-fold with K_2HPO_4 (10 mM), pH 8.0, and loaded onto a mini-Q anion-exchange column (Amersham Pharmacia), equilibrated in K_2HPO_4 (14 mM), pH 8.0, containing NaCl (0.1 M). The [^{33}P]CoA was eluted isocratically with K_2HPO_4 (14 mM), pH 8.0, containing NaCl (0.1 M), at a flow rate of 1 mL/min and freeze-dried.

^{33}P Isotope Exchange ([^{33}P]CoA to Acetyl-[^{33}P]CoA]) at Equilibrium. Four reactions (250 μL) containing SAT (0.24 μM), [^{33}P]CoA (1 mM), and AcCoA (0.04, 0.1, 0.3, and 0.7 mM) in TSE buffer were incubated at 25 °C. Aliquots (50 μL) were removed at 0, 5, 10, 20, and 30 min intervals and frozen. Thawed aliquots were mixed with thiol−Sepharose 4B (50 μL) and equilibrated in TSE buffer, for 1 min, and unbound AcCoA was eluted with four aliquots (200 μL each) of TSE buffer. Bound CoA was eluted with cysteine (0.1 M) in TSE buffer (4 × 200 μL as above). Samples were counted in OptiPhase "HiSafe" 3 scintillant (Perkin-Elmer) in a Packard 2100TR Tri-Carb liquid scintillation analyzer.

Direct Spectrophotometric Assay. Kinetic measurements in the presence of added thiols were performed by monitoring the net change in absorbance at 232 nm ($\epsilon^M = -4.2 \times 10^3$ $cm^{-1}\cdot M^{-1}$) for the forward reaction catalyzed by SAT (*1*). Reactions were initiated by the addition of 10 μL of an appropriately diluted stock of SAT (in TSE buffer) to a solution of final volume 1 mL, containing TSE buffer, substrates, and the thiol of interest.

Inhibition Analysis of the Forward Reaction. Apart from alanine, which was varied over the concentration range 0.2−1.5$K_{iA'}$, each inhibitor was varied from 0.33K_i to 5K_i; the fixed substrate concentration was set at or near K_m, and the nonfixed substrate was varied from 0.33K_m to 5K_m.

Product Inhibition. Product inhibition analyses were performed over a matrix of four concentrations of serine over the range 0.33−4K_m and four fixed concentrations of CoA (0.33−3K_m) in the presence of AcCoA (50 μM).

Stopped-Flow Kinetics. An SF-17MV stopped-flow spectrophotometer (Applied Photophysics, Leatherhead, U.K.) with a dead time of 2 ms was used. The syringes were driven by a pneumatic actuator operated by compressed nitrogen (pressure from 5 to 8 bar). All reactions were performed in TSE buffer at 25 °C in 0.2 cm path length cells. The coupled DTNB assay was utilized to monitor the release of CoA upon hydrolysis of AcCoA by SAT. Typically three traces were collected for signal averaging and storage on computer disk. Data acquisition and processing were controlled by a 32-bit processor Archimedes workstation.

Microcalorimetric Titration. Complete binding isotherms were obtained at 25 °C in TSE buffer in a Microcal Omega titration microcalorimeter. Raw data were collected for an automated sequence of injections (*20*−*41*), each of 1−2 μL, spaced at 4 min intervals. The duration of each injection was 5 s. Data were corrected for ligand heats of dilution and then deconvoluted using an algorithm described previously (*31*).

Direct Determination of K_{eq}. A 10 μL aliquot of SAT (48 μM, in TSE buffer) was added to a solution of final volume 1 mL and containing *O*-acetylserine (6.95 mM), serine (0.245 mM), and AcCoA (0.14 mM) in TSE buffer. The equilibrium concentration of AcCoA and the corresponding stoichiometric changes in concentration of serine and each product were estimated by measuring the change in absorbance at 232 nm, in triplicate, at 25 °C over an interval in which AcCoA hydrolysis was insignificant (<30 s) and acetyl transfer was no longer detectable. The data were used to calculate the equilibrium constant (K_{eq}) for the forward reaction:

$$K_{eq} = [CoA][O\text{-acetylserine}]/[serine][AcCoA]$$

RESULTS AND DISCUSSION

Purification of SAT. The purified enzyme showed no *O*-acetylserine (thiol)-lyase activity and migrated in SDS−PAGE as a sharp band with no detectable contaminating proteins (data not shown). The present purification protocol (*18*), which is a modification of that of Wigley and co-workers (*9*), gives a significant improvement in yield and a 10-fold increase of the final specific activity of SAT (719 units/mg), which compares very favorably with published values for SAT from *S. typhimurium* [97 units/mg (*32*) and 70 units/mg (*6*)].

Accelerative Initial Rates. Although the most highly purified preparations of SAT, which were used for all the experiments reported below, produced a fully linear initial rate under the standard assay conditions, the less highly purified preparations exhibited an acceleration in initial rates for the first 2−3 min of measurement. Completely linear initial rates could be conferred on such fractions by preincubation for 2−3 min with DTNB (1 mM), an effect previously reported by Leu and Cook (*6*). Furthermore, the most highly purified SAT, which showed no detectable reaction with DTNB, as judged by absorbance measurements at 412 nm (data not shown), was also stabilized upon dilution (1−5 μM) into TSE buffer by DTNB (1 mM). We propose that the apparent activation of partially purified SAT involves a reaction between DTNB and trace amounts of an inhibiting contaminant thiol, which has yet to be identified, whereas stabilization of dilute stocks of the most highly purified SAT involves a noncovalent interaction of DTNB with the enzyme.

Initial Velocity Studies. All steady-state kinetic analyses of the forward reaction were performed in the presence of subinhibitory concentrations of serine (8 mM) and yielded linear Lineweaver−Burk plots (Figure 1a). Under the experimental conditions employed for the forward reaction a statistically reliable ϕ_{AB} term (see below) was detected, which is consistent with a ternary complex mechanism but not compatible with a double-displacement mechanism (*26*). Furthermore, the presence of ethyl-*S*-CoA (72 μM), a competitive inhibitor with respect to AcCoA, resulted in a 2.4-fold increase in ϕ_{AB} and a 1.8-fold increase in ϕ_B (Table 1), a result in keeping with either a random-order or a compulsory-order ternary complex mechanism in which B (acetyl donor) leads but inconsistent with either a double-displacement or a compulsory-ordered sequential mechanism in which A (acetyl acceptor) leads.

Substrate Inhibition of Acetyl Transfer to Serine. The inhibition of acetyl transfer, over a 15-fold range of

Table 1: Kinetic Coefficients, at 25 °C and pH 7.5, for the (i) Forward and (ii) Reverse Reactions[a]

forward reaction	$10^3\phi_o$ (s)	$10^3\phi_A$ (s·mM)	$10^3\phi_B$ (s·mM)	$10^3\phi_{AB}$ (s·mM)	ϕ_A/ϕ_o (mM) $K_{A(B)}$	ϕ_B/ϕ_o (mM) $K_{B(A)}$	ϕ_{AB}/ϕ_A (mM) K_B	ϕ_{AB}/ϕ_B (mM) K_A	$1/\phi_o$ (s^{-1})
(i) AcCoA + serine	2.35 ± 0.11	2.75 ± 0.18	0.45 ± 0.06	0.335 ± 0.065	1.17	0.2	0.13	0.75	427
(i) AcCoA + serine + EtCoA	2.10 ± 0.17	2.90 ± 0.25	0.82 ± 0.15	0.8 ± 0.14					
(i) AcCoA (thioesterase)	14710					0.146	0.146		0.068

reverse reaction	$10^3\phi_{o'}$ (s)	$10^3\phi_P$ (s·mM)	$10^3\phi_Q$ (s·mM)	$10^3\phi_{PQ}$ (s·mM)	$\phi_P/\phi_{o'}$ (mM) $K_{P(Q)}$	$\phi_Q/\phi_{o'}$ (mM) $K_{Q(P)}$	ϕ_{PQ}/ϕ_P (mM) K_Q	ϕ_{PQ}/ϕ_Q (mM) K_P	$1/\phi_{o'}$ (s^{-1})
(ii) CoA + *O*-acetylserine	59.3 ± 8.1	424 ± 72	4.76 ± 0.95	60.5 ± 0.75	7.15	0.08	0.14	12.7	16.9

[a] The kinetic coefficients are those in the initial rate equation, where A is serine and B is AcCoA. For the reverse reaction P is *O*-acetylserine and Q is CoA. ϕ_A/ϕ_o ($K_{A(B)}$) and $\phi_P/\phi_{o'}$ ($K_{P(Q)}$) are the Michaelis constants (K_m) for serine and *O*-acetylserine, respectively, and ϕ_B/ϕ_o ($K_{B(A)}$) and $\phi_Q/\phi_{o'}$ ($K_{Q(P)}$) are the Michaelis constants (K_m) for AcCoA and CoA, respectively.

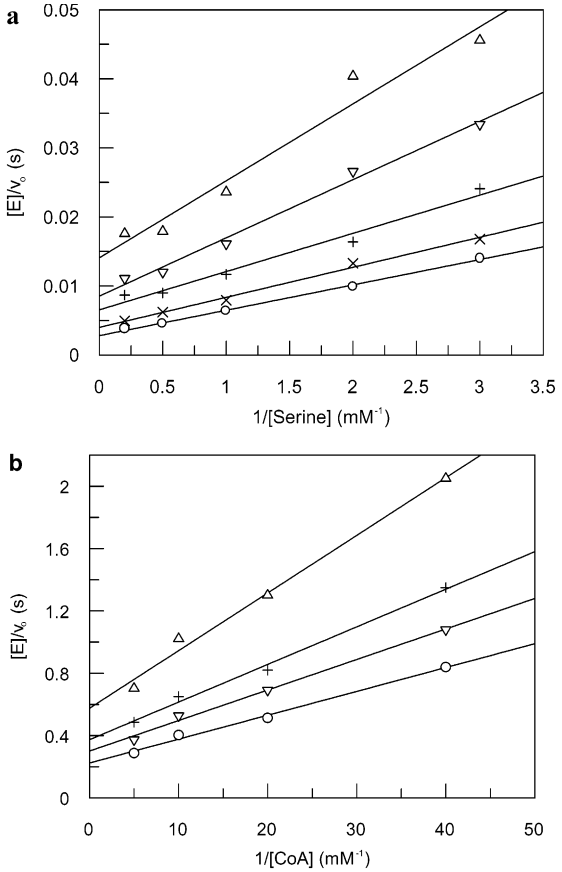

FIGURE 1: Double-reciprocal plots for SAT showing forward and reverse reaction data. (a) Lineweaver−Burk plot for the acetylation of serine by SAT over a range of serine and fixed AcCoA concentrations. The final enzyme (monomer) concentration was 1.7 nM, and the incubation conditions were as described in Experimental Procedures (pH 7.5 and 25 °C). Key: (○) 0.6 mM AcCoA, (×) 0.25 mM AcCoA, (+) 0.12 mM AcCoA, (▽) 0.07 mM AcCoA, and (△) 0.04 mM AcCoA. (b) Lineweaver−Burk plot for the acetylation of CoA by SAT over a range of *O*-acetylserine and fixed CoA concentrations. The final enzyme (monomer) concentration was 25.7 nM, and the incubation conditions were as described in Experimental Procedures (pH 7.5 and 25 °C). Key: (○) 8 mM *O*-acetylserine, (▽) 5 mM *O*-acetylserine, (+) 3.5 mM *O*-acetylserine, and (△) 2 mM *O*-acetylserine.

concentrations of AcCoA (from $0.33K_m$ to $5K_m$), by concentrations of serine greater than 8 mM (data not shown) is consistent with the accumulation of a kinetically significant but a nonproductive ternary complex (enzyme·CoA·serine). Such a complex is diagnostic for a ternary complex mechanism in which substrate addition is random or in which AcCoA leads. The latter is ruled out by the results of dead-end inhibition studies described below.

Table 2: Dead-End Inhibitor Dissociation Constants for SAT[a]

inhibitor (A)	binary (mM) $K_{iA'}$	ternary (mM) $K_{iA'(B)}$	inhibitor (B)	binary (mM) $K_{iB'}$	ternary (mM) $K_{iB'(A)}$
glycine	13.6 ± 4.3	7.6 ± 1.2	ATP	13.4	6.7
alanine	272	66			

[a] Binary and ternary complex dead-end inhibitor dissociation constants were derived from gradient and intercept replots, of primary double-reciprocal plots, according to the rapid-equilibrium random-order assumption for a ternary complex mechanism. Data for glycine are the mean ± standard deviation for three separate measurements, whereas those for alanine and ATP are for a single kinetic measurement.

Confidence in Kinetic Parameters Derived from ϕ Coefficients. Before ϕ coefficient relationships were analyzed as indicators of specific mechanisms, the reliability of each coefficient was evaluated by calculating its percentage contribution to the overall rate equation, under the conditions in which it is most likely to dominate (*20*, *33*). For example, for the forward reaction, ϕ_o is most likely to dominate at the highest substrate concentrations employed (5 mM serine and 1.08 mM AcCoA). Such proved to be the case, since the calculated percentage contribution of each term to the rate equation, under such conditions, showed ϕ_o to be the major contributor (ϕ_o, 69%, with lesser values for the other coefficients: ϕ_A/[A], 16%; ϕ_B/[B], 13%; ϕ_{AB}/[A][B], 2%).

Dead-End Inhibition of Acetyl Transfer to Serine. Glycine and alanine (Table 2) are mixed noncompetitive inhibitors, with respect to AcCoA, whereas glycine is a competitive inhibitor with respect to serine. Such a pattern of inhibition should not be observed with an ordered sequential mechanism for SAT in which AcCoA leads but is consistent with (a) an ordered mechanism in which serine leads or (b) a rapid-equilibrium random-order mechanism (*33*). EtCoA (data not shown) and ATP (data not shown) are competitive inhibitors with respect to AcCoA whereas ATP (data not shown) is a mixed noncompetitive inhibitor with respect to serine. An ordered reaction mechanism in which serine leads can therefore be ruled out. The overall pattern of dead-end inhibition is therefore consistent with a random order of substrate addition. Moreover, the linearity of the double-reciprocal plots and the secondary replots is as expected for a rapid-equilibrium random-order mechanism, suggesting that interconversion of ternary complexes may be partially rate-determining.

[^{14}C]Serine/O-Acetylserine Isotope Exchange at Equilibrium. Although thin-layer chromatography (data not shown) revealed acetylation of [^{14}C]serine by AcCoA in a control reaction, *O*-acetyl-[^{14}C]serine was not detected in isotope exchange assays, indicating that *O*-acetylserine is not in enzyme-catalyzed equilibrium with [^{14}C]serine. Hence, the reaction mechanism cannot involve the formation and breakdown of an acetyl-enzyme intermediate, thereby ruling out a double-displacement reaction for SAT from *E. coli*, the mechanism proposed by Leu and Cook (*6*) for SAT from *S. typhimurium*.

[^{33}P]CoA/AcCoA Isotope Exchange at Equilibrium. [^{33}P]-CoA was purified by anion-exchange chromatography (data not shown). Equilibrium isotope exchange experiments showed that there was no increase with time in radioactivity attributable to AcCoA, ruling out isotope exchange between [^{33}P]CoA and AcCoA (data not shown). Thioesterase activity of SAT could not have prevented the accumulation of [^{33}P]-AcCoA since control experiments showed <10% of the total AcCoA to be depleted during the course (30 min) of a typical exchange experiment (data not shown). The data accommodate a ternary complex mechanism for SAT but not one that proceeds by a double-displacement reaction involving a covalent acetyl-enzyme intermediate.

Kinetic Deductions for a Rapid-Equilibrium Random-Order Mechanism. Each binary complex dissociation constant (K_s) for SAT was derived according to Dalziel and Dickenson (*34*) for a rapid-equilibrium and random-order pattern of substrate addition. For such binary complexes the observed dissociation constant (K_s) for each substrate is lower than its counterpart (K_m) for the ternary complex (Table 1). As such, there must be a decrease in the affinity of SAT for each of its substrates during the transition from a binary to a productive ternary complex, a phenomenon observed with chloramphenicol acetyltransferase, another member of the trimeric family of bacterial acetryltransferases (*13*) and described as "negative cooperativity" (*29*). Such a decrease in affinity of SAT for serine and AcCoA in the ternary complex, which would amount to a free energy change ($\Delta G_c°$) of 0.3 kcal·mol^{-1}, may in effect be a measure of the phenomenon referred to as "strain" (*35*), which attempts to describe a lessening of "binding only" interactions in the approach to the transtion state, where new contacts and geometries favor catalysis.

Dissociation Constants for Dead-End Inhibitors. The interaction of a dead-end inhibitor with an enzyme obeying a rapid-equilibrium random-order reaction mechanism yields binary and ternary nonproductive complexes that are at equilibrium and thus from which dead-end inhibitor dissociation constants may be determined kinetically (Table 2). It has also been shown that enzymes for which the central step is partially rate-limiting, such as SAT, can be treated similarly (*36*).

Thioesterase Activity of SAT: Stopped-Flow Kinetics. A linear rate of CoA release, without a "burst" phase, was observed upon mixing excess AcCoA with SAT, and a similar result was obtained with PrCoA (data not shown). Data for the hydrolytic turnover of a substoichiometric concentration of AcCoA were fitted to a single exponential with a floating end point (Figure 2). Although the initial rate observed for CoA release (0.034 s^{-1}) was 4 orders of magnitude below k_{cat} for acetyl transfer to serine, under steady-state conditions, it approaches that of k_{cat} for AcCoA hydrolysis (0.068 s^{-1}) measured under steady-state conditions (Table 1).

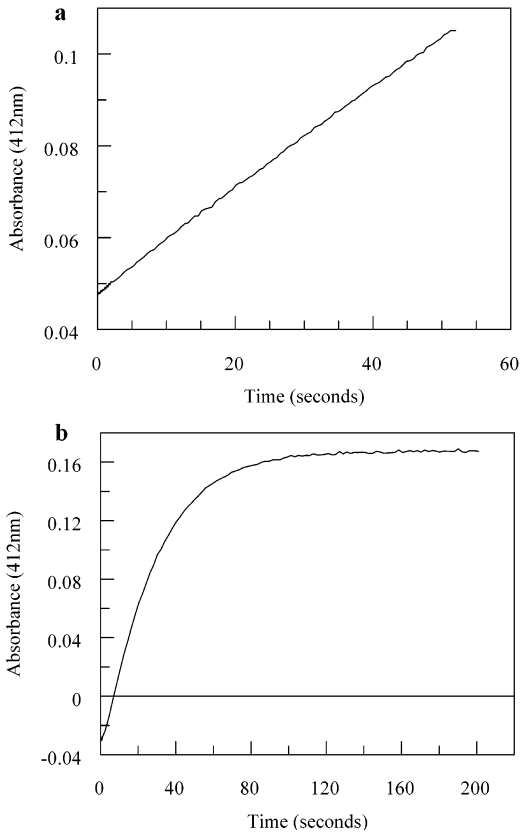

FIGURE 2: Pre-steady-state kinetic analysis of the thioesterase activity of SAT. (a) Experiment with a saturating concentration of AcCoA. Syringe A contained AcCoA (280 μM) and DTNB (1 mM) in TSE buffer. Syringe B contained SAT (13.1 μM) and DTNB (1 mM) in TSE buffer. The cell was blanked with TSE buffer containing DTNB (1 mM). All reactions were performed at 25 °C as described in Experimental Procedures. The release of CoA was detected by its reaction with DTNB and absorbance increase at 412 nM. The observed monophasic (linear) initial rate of AcCoA hydrolysis indicates that the turnover does not involve a kinetically significant chemical step or, were there to be a biphasic release of CoA, the expected initial burst must be too rapid to measure. Moreover, since a control experiment without SAT produced a background absorbance change of 0.04 at 412 nM (attributed to a trace amount of CoA), the net absorbance change upon mixing enzyme with AcCoA is less than 0.01. This is unlikely to comprise a contribution from protein absorbance and a burst of CoA release, since the latter is expected to contribute 0.018 absorbance unit at 412 nM. (b) Experiment with a nonsaturating concentration of AcCoA. Syringe A contained AcCoA (160 μM) and DTNB (1 mM) in TSE buffer. Syringe B contained SAT (212 μM) and DTNB (1 mM) in TSE buffer. All reactions were performed at 25 °C as described in Experimental Procedures. The rate of AcCoA hydrolysis is 4 orders of magnitude too slow for it to constitute a committed step on a double-displacement reaction mechanism (see Discussion).

Reverse Reaction. Steady-state kinetic analyses of the reverse reaction yielded linear Lineweaver−Burk plots (Figure 1b) when carried out in the presence of subinhibitory concentrations of *O*-acetylserine (11 mM) and CoA (200 μM). Over the duration of a typical kinetic analysis it could be calculated (*38*) that less than 1% of *O*-acetylserine underwent tautomeric rearrangement to *N*-acetylserine. Moreover, since *N*-acetylserine produced no inhibition in a standard SAT assay at concentrations as high as 30 mM, its

presence could not have affected the reverse reaction. Under the experimental conditions employed, $\phi_{o'}$ did not make a major contribution to the overall rate equation (Table 1). The presence of a significant ϕ_{PQ} term indicates that the reverse reaction proceeds via a ternary complex mechanism. Moreover, substrate inhibition by CoA is consistent with the accumulation of a kinetically significant but a nonproductive ternary complex (enzyme·CoA·serine). Taken together, the data are consistent with either (a) a steady-state random-order process or (b) an ordered mechanism with *O*-acetylserine leading. The observed inhibition at high concentrations (>11 mM) of *O*-acetylserine (~95% pure, containing small amounts of serine and *N*-acetylserine) could be a consequence of either (a) product inhibition by serine or (b) the formation of a nonproductive ternary complex (enzyme·AcCoA·*O*-acetylserine).

Direct Determination of the Equilibrium Constant. The directly determined equilibrium constant (*58*) for the reaction catalyzed by SAT (see Experimental Procedures) corresponds to a free energy change of -2.4 kcal·mol^{-1}. Moreover, the experimental attainment of equilibrium was sufficiently rapid (<1 min) to prevent a significant error due to AcCoA hydrolysis. The free energy change in transferring acetyl from CoA to serine approximates to that determined for transfer to chloramphenicol, another primary alcohol (-2.1 kcal·mol^{-1}; *20*), whereas the transfers to choline (-1.5 kcal·mol^{-1}) or carnitine (-0.3 kcal·mol^{-1}) are known to be less exergonic (refs *39* and *40*, respectively).

Microcalorimetrically and Kinetically Derived Dissociation Constants. Since SAT exhibits no change in intrinsic fluorescence on the addition of substrates, substrate analogues, or products (data not shown), it is not possible to determine dissociation constants fluorometrically. Neither could the dissociation constant of *O*-acetylserine, nor that of AcCoA, be determined by substrate depletion or microcalorimetry, since the former is tautomeric and SAT catalyzes the hydrolysis of the latter.

However, it was possible to measure the dissociation constant for the SAT·CoA binary complex ($K_d = 213$ μM) from the inhibition of the forward reaction by CoA (which is a mixed noncompetitive inhibitor with respect to serine) and independently by microcalorimetry ($K_d = 148$ μM). Both values are in reasonable agreement with the kinetically derived steady-state estimate (143 μM) for either (a) a rapid-equilibrium random-order or (b) an ordered sequential mechanism in which CoA leads (Table 1). Furthermore, since substrate inhibition of the reverse reaction by CoA is consistent with the formation of a SAT·serine·CoA dead-end ternary complex, it follows that an ordered mechanism in which CoA leads can be ruled out. The intrinsic thioesterase activity of SAT indirectly leads to an estimate of K_s for AcCoA in the form of a Michaelis constant for hydrolysis. The following basic kinetic scheme for SAT and a single substrate assumes that k_{+2} is the rate-limiting step for the overall reaction, the evidence for which is at hand:

$$\text{SAT} + \text{AcCoA} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{SAT·AcCoA} \xrightarrow{k_{+2}} \text{SAT} + \text{CoA} + \text{acetate}$$

Since $k_{+2} = 0.068$ s^{-1}, as determined by steady-state measurements of the thioesterase activity (data not shown) and the observation that coenzyme dissociation rate constants are normally in the range of $10-10^3$ s^{-1} (*41*), it follows that $k_{+2} \ll k_{-1}$ and hence that K_m reduces to K_s. The K_m value for AcCoA (146 μM) hydrolysis (data not shown) is in good agreement with the kinetically derived K_s value for acetyl transfer (123 μM), as expected for a rapid-equilibrium random-order mechanism. Further evidence that both the acetyltransferase and acetylthioesterase activities are both intrinsic properties of SAT, rather than the latter being a contaminating hydrolase, include (a) the copurification of the two activities by an affinity method and (b) their concordant sensitivity to inhibition by glycine (reversible) and irreversible inhibition by iodoacetamide (data not shown).

SUMMARY

The kinetic studies presented above are consistent with a steady-state random-order mechanism for SAT from *E. coli*, one in which the breakdown of the enzyme·CoA and the enzyme·serine complexes is partially rate-determining for the forward and the reverse directions, respectively. Hence, SAT is mechanistically similar to galactokinase from *E. coli* (*36*), which was shown to follow a steady-state random-order mechanism, notwithstanding reciprocal plot data and product inhibition results that are indistinguishable from those expected for a rapid-equilibrium random-order mechanism.

The most striking conclusion from the present experiments is that highly purified SAT from *E. coli* follows a sequential (ternary complex) mechanism, rather than a double-displacement one, as proposed by Leu and Cook (*6*) on the basis of experiments with the homologous protein from *S. typhimurium*. Although the near identity of the two bacterial primary SAT structures, as well as conclusions from studies of the *lacA* (*11*) and *nodL* (*16*, *17*) gene products, is a strong argument that members of this small subfamily of trimeric enzymes catalyze *O*-acyl transfer by a common mechanism, a compelling explanation for the discrepancy between our conclusions and those of Leu and Cook is not yet at hand. Nonetheless, we believe that the data presented here, and use of the "principle of parsimony" to guide thinking in evolutionary biology, argue that not only SAT from *E. coli* but also its nearly identical homologue from *S. typhimurium* catalyzes the first step in the synthesis of cysteine by a common kinetic and chemical mechanism, one which involves a productive ternary complex of substrates and enzyme.

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