Serine acetyltransferase of *Escherichia coli*: substrate specificity and feedback control by cysteine

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Although SAT (serine acetyltransferase) of *Escherichia coli*, which catalyses the first step in cysteine synthesis, proceeds via a random-order ternary complex reaction mechanism [Hindson and Shaw (2003) Biochemistry 42, 3113–3119], it has been suggested that the nearly identical enzyme from *Salmonella typhimurium* might involve an acetyl-enzyme intermediate [Leu and Cook (1994) Protein Peptide Lett. 1, 157–162]. In this study the alternative acetyl acceptor threonine and the alternative acyl donor, propionyl-CoA were used to further investigate the reaction mechanism of SAT from *E. coli*. Steady-state kinetic data and dead-end inhibition studies were again diagnostic of a random-order ternary complex reaction mechanism for alternative substrates. Since earlier kinetic studies with SAT from *S. typhimurium* suggested that cysteine competes with acetyl-CoA for binding, rather than serine with which it is isostructural, the site is unlikely to provide obligatory short-range contacts with the hydroxyl group of serine, a prerequisite for exclusion of cysteine. Such a proposal is supported by the results of micro-calorimetric studies which show that cysteine competes with serine for binding to SAT rather than with CoA. It follows that tight binding of cysteine at the serine-binding site near the catalytic centre may be the effector of a substantial reduction in the affinity of SAT for CoA, yielding the observed pattern of steady-state inhibition and the mechanism by which cysteine mediates effective end-product control of its synthesis.

Key words: acyl transfer, coenzyme A (CoA), cysteine, calorimetry.

**INTRODUCTION**

SAT (serine acetyltransferase; EC 2.3.1.30) catalyses the O-acetylation of serine, the first reaction in the two-step process of sulphur assimilation by micro-organisms [1–3] and higher plants [4,5]. The second step, cysteine synthesis, is catalysed by O-acetylserine (thiol)-lyase (EC 2.5.1.47).

Serine + AcCoA → O-acetyl-serine + CoA (1)

O-acetyl-serine + sulphide → cysteine + acetate (2)

where AcCoA is acetyl-CoA. In both *Salmonella typhimurium* and *Escherichia coli* it has been observed that SAT is associated reversibly with approx. 5% of the total cellular O-acetylserine (thiol)-lyase to form the multi-enzyme complex referred to as cysteine synthase, and that the flow of substrates through this pathway is regulated by cysteine, a negative-feedback inhibitor of SAT [2]. Surprisingly, cysteine has been reported to be a competitive inhibitor with respect to AcCoA [1,2], rather than with serine, with which it is isostructural, indicating that cysteine binds preferentially at the coenzyme-binding site rather than the serine-binding site. Furthermore, a comparison of the apparent $K_m$ values for serine (0.77 mM) and AcCoA (100 µM) with the apparent $K_i$ for cysteine (1 µM) showed that SAT of *S. typhimurium* binds cysteine with relatively high affinity [6]. Such findings have lead to speculation that cysteine might bind at an allosteric site on SAT, concomitantly lowering the affinity of SAT for AcCoA [7].

Forms of SAT from *S. typhimurium* and *Spinacia oleracea* have been reported to follow a double-displacement (Ping Pong) mechanism [8–10], whereas the *E. coli* variant follows a random-order ternary complex mechanism [11]. The present study was undertaken to study the reaction mechanism of SAT in greater detail and, more particularly, to elucidate the mechanism of inhibition by cysteine. The results of the kinetic studies presented below are consistent with the view that SAT of *E. coli*, like each of the trimeric microbial O-acetyltransferases [12,13] studied thus far, apart from SAT from *S. typhimurium* and *S. oleracea*, share a common kinetic mechanism that involves a productive ternary complex of substrates and enzyme. Furthermore, micro-calorimetric data indicate that cysteine binds with high affinity at the serine site of *E. coli* SAT, lowering the affinity of SAT for CoA. It follows that tight binding of cysteine at the serine-binding site near the catalytic centre may be the effector of a substantial reduction in the affinity of SAT for CoA, yielding the observed pattern of steady-state inhibition and the mechanism by which cysteine mediates end-product control of its synthesis. Furthermore, such a mechanism may have evolved to preclude the formation of a productive SAT–cysteine–AcCoA ternary complex, which could give rise to the acetylation of cysteine.

Abbreviations used: AcCoA, acetyl-CoA; PrCoA, propionyl-CoA; SAT, serine acetyltransferase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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**MATERIALS AND METHODS**

**Chemicals**
All chemicals were of 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 harbouring pSAT3 that contains the gene for ampicillin resistance and the *cySE* gene that encodes for SAT [14], as described by Hindson and co-workers [15]. The purification protocol was a modification of that of Wigley et al. [16], which gives both a significant improvement in yield and a 10-fold increase in the final specific activity of SAT (719 units/mg). All kinetic studies were performed on a single preparation of SAT (719 units/mg).

**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, less highly purified preparations exhibited an acceleration in initial rates for the first 2–3 min of measurement. Completely linear initial-rate behaviour could be conferred on such fractions by preincubation for 2–3 min with DTNB [5,5′-dithiobis(2-nitrobenzoic acid; 1 mM), as described and discussed by Hindson and Shaw [11].

**Preparation of acyl-CoA**
AcCoA and PrCoA (propionyl-CoA) were synthesized by the method of Simon and Shemin [17] as described by Kleanthous and Shaw [18].

**SAT standard assay**
The standard assay mixture contained TSE buffer (50 mM Tris/HCl, pH 7.5, containing 0.1 M NaCl and 0.1 mM EDTA), 1 mM DTNB, 0.8 mM AcCoA and 5 mM serine in a volume of 990 µl. The reaction was initiated by the addition of 10 µl of enzyme in TSE buffer containing DTNB (1 mM) and product formation was monitored by measuring the increase in absorbance at 412 nm due to the reaction of CoA with DTNB. Rates were measured by using an absorption coefficient for 5-thio-2-nitrobenzoate at 412 nm, of 1.36 × 10^4 cm^−1·M^−1. Stock solutions of SAT were made up in TSE buffer containing 1 mM DTNB and were stable over the duration of a typical 1 h kinetic analysis. Unless otherwise stated kinetic analyses were performed over a matrix of at least four substrate concentrations (for acyl donor and acceptor), in the range 0.33 × *K_m*–5 × *K_m*.

Since the kinetic data for the forward reactions yielded linear Lineweaver–Burk plots (eqn 3) over the entire concentration range of substrates utilized the rapid-equilibrium assumption could be applied [19,19a,20]. Kinetic coefficients in the Hanes rate equations (eqn 4) were derived according to Dalziel [21] from linear intercept and slope replots from computer-fitted Hanes plots (eqn 4).

\[
E/V_o = \Phi_o + \Phi_A/[A] + \Phi_B/[B] + \Phi_{AB}/[A][B] 
\]

where \( E \) is the concentration of total enzyme, \( V_o \) is the initial rate of product formation, \( A \) is the acyl acceptor substrate, \( B \) is the acyl donor substrate and each \( \Phi \) coefficient is a compound rate-constant term for the forward reaction.

**\( K_m \) and \( k_i \) for the hydrolysis of PrCoA**
Conditions were identical with those of the forward reaction assays, except that no serine was incorporated and the concentration of SAT was typically in the range 1–5 µM. \( K_m \) and \( k_i \) were determined by direct linear plots [22].

**Cysteine inhibition**
The reaction was monitored by measuring the net change in absorbance at 232 nm (\( \varepsilon = -4.2 \times 10^4 \text{ cm}^{-1} \cdot \text{M}^{-1} \)) for the forward reaction catalysed by SAT [8]. 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. Data were collected over three or four concentrations of cysteine (up to 5 × *K_m*), where \( K_m \) is the binary complex inhibitor dissociation constant for substrate analogue of A) in the presence of either: (i) a fixed concentration of AcCoA (\( K_m \)) and four concentrations of serine in the range 0.33 × *K_m*–4 × *K_m* or (ii) a fixed concentration of serine (2 × *K_m*–3 × *K_m*) and four concentrations of AcCoA in the range 0.2 × *K_m*–1 × *K_m*.

**Inhibition analyses of the forward reaction**
All assays were based on the standard SAT assay and were performed in triplicate at 25 °C in a final volume of 1 ml. Each inhibitor was varied from 0.33 × *K_m* to 5 × *K_m*; the fixed substrate concentration was set at or near *K_m* and the non-fixed substrate was varied from 0.33 × *K_m* to 5 × *K_m*.

**Micro-calorimetric titration**
Complete binding isotherms were obtained at 25 °C in TSE buffer in a Microcal Omega titration micro-calorimeter. Raw data were collected for an automated sequence of injections (20–45), 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 [23].

Apparent dissociation constants for cysteine (\( K_{cy} \)) were calculated according to eqn (5), in which \( K_{cy} \) is the dissociation constant for cysteine and \( K_s \) is the dissociation constant of the candidate competitive inhibitor.

\[
K_{cy} = K_s(1 + [X]/K_s) 
\]
RESULTS AND DISCUSSION

Confidence in kinetic parameters derived from \( \Phi \) coefficients

The reliability of each \( \Phi \) coefficient was evaluated by calculating whether its compound rate equation term provides the greatest percentage contribution to the overall rate equation at the substrate concentrations that yield its maximum value [18,24]. For example, for the acetylation of threonine, the \( \Phi_A \) term will attain its maximum value at the highest concentration of substrates employed (500 mM threonine and 1.3 mM AcCoA). The data, however, show the percentage contribution of the \( \Phi_A \) term (\( \Phi_A/[A] \), 66.4%) to be approx. four times as great as that of the \( \Phi_o \) term (\( \Phi_o \), 17.8%; with values for the other \( \Phi \) terms being 10.2% for \( \Phi_B/[B] \) and 5.6% for \( \Phi_{AB}/[A][B] \)) under such conditions, indicating that \( \Phi_o \) may include a small error.

Kinetic analyses with alternative substrates

A kinetic approach similar to that employed by Kleanthous and Shaw [18] in studies with chloramphenicol acetyltransferase was used to study the effects of alternative substrates on the kinetic coefficients (Figures 1 and 2). Data were compared with those for the acetylation of serine [11]. The concentration of threonine was varied from 0.01 \( K_m \) to 0.35 \( K_m \), that of AcCoA from 0.07 \( K_m \) to 2 \( K_m \), PrCoA from 0.3 \( K_m \) to 1.5 \( K_m \), and serine from 0.2 \( K_m \) to 0.75 \( K_m \). Butyryl-CoA at 1 mM was turned over 10\(^3\)-fold more slowly than AcCoA, making it unsuitable for a useful kinetic analysis (results not shown). In all such experiments \( \Phi_A \), \( \Phi_B \) and \( \Phi_{AB} \) made a dominant contribution to the overall rate equation, whereas \( \Phi_o \) made a major contribution. The latter was therefore deemed to be less reliable.

The observed 3.4-fold increment in \( \Phi_o \) for the acetylation of threonine is inconsistent with an ordered sequential mechanism in which AcCoA leads, wherein \( 1/\Phi_o \) is the on rate \( (k_o) \) for AcCoA (the leading substrate) and is independent of variation in the structure of the inner substrate (Table 1). The ratio \( \Phi_{AB}/\Phi_o \) is the dissociation constant of AcCoA from free enzyme for either (i) a rapid-equilibrium random-order mechanism or (ii) an ordered mechanism in which AcCoA leads. Hence the substantial agreement between the values of \( \Phi_{AB}/\Phi_o \) for threonine and serine is consistent with a random order of substrate addition. The good agreement between \( \Phi_o \) for the acetylation of threonine and serine suggests that \( k_o \) \( (1/\Phi_o) \) is unchanged and hence that the rate-limiting step is conserved for both reactions. However, as noted above, \( \Phi_o \) is judged to be less reliable.

The 17-fold increment in \( \Phi_A \) (over that for AcCoA) for the acetylation of serine by PrCoA demonstrates that the length of the acyl chain has an adverse effect on the rate-determining step, either by increasing the activation energy for catalysis or by decreasing the off rate of \( O \)-propionyl-serine (or CoA) from the ternary complex (Table 1). The lack of inhibition of propionyl transfer by serine indicates that a dead-end ternary complex (enzyme–CoA–serine) does not accumulate (results not shown). Hence the dissociation of CoA from the binary complex of enzyme and CoA cannot be rate-determining. The observed 115-fold increment in \( \Phi_A \), for propionyl transfer convincingly rules out an ordered (either sequential or Theorell–Chance) mechanism in which serine leads, since under such a mechanism \( 1/\Phi_o \) corresponds to the on rate \( (k_o) \) for serine (first substrate) and should be independent of changes in the structure of the acyl donor. The 4-fold variation in the term \( \Phi_{AB}/\Phi_B \), which is the dissociation constant of serine from free enzyme, for either a rapid-equilibrium random-order mechanism, or an ordered mechanism with serine leading, was surprising, given that acetyl transfer to serine approximates closely to a rapid-equilibrium random-order mechanism. The discrepancy could, therefore, be a consequence of the breakdown of the rapid-equilibrium assumption for the faster acetyl-transfer reaction, rather than evidence that propionyl transfer does not follow such a pathway.

The 90-fold lower specificity of SAT towards PrCoA, as shown by \( k_o/K_m \), indicates that its additional methylene group compromises binding at the AcCoA site. Such a notion is supported by the inability of butyryl-CoA at 1 mM to serve as an alternative acyl donor or to inhibit acetyl transfer in a standard SAT assay (results not shown). The acyl-CoA-binding site thus appears to have evolved to discriminate against higher acyl homologues of AcCoA to limit the inappropriate acylation of serine in vivo. Although not studied in the present series of experiments, it is of
interest that formyl-CoA has been reported to be an acyl donor
in vitro [1].

Dead-end inhibition of propionyl transfer to serine

Glycine (Figure 3) is a mixed non-competitive inhibitor of propionyl transfer to serine with respect to PrCoA. Such a pattern of inhibition should not be observed with an ordered (Theorell–Chance, ordered sequential or double-displacement) reaction mechanism for SAT in which PrCoA leads, but is consistent with (i) an ordered mechanism in which serine leads or (ii) a rapid-equilibrium random-order mechanism. ATP (Figure 4) is a mixed non-competitive inhibitor with respect to serine, ruling out an ordered reaction mechanism in which serine leads. The overall pattern of dead-end inhibition is therefore consistent with random-order substrate addition. Moreover, the absence of substrate inhibition by serine and the linearity of primary double-reciprocal plots suggests that the interconversion of ternary complexes is at least partially rate-limiting, favouring a rapid-equilibrium random-order mechanism over a steady-state random-order one.

Dead-end inhibitor dissociation constants

Binary complex dissociation constants for glycine (5 mM) and ATP (19 mM) were derived by applying the rapid-equilibrium assumption to the propionyl transfer reaction. These values agreed quite well with those for glycine (14 mM) and ATP (13.5 mM) derived by applying the same assumption to the steady-state random-order serine acetyl-transfer reaction [11]. Using the approach of Gulbinsky and Cleland [25], analysis of the data therefore supports the proposal that acetyl transfer to serine involves a rate-determining step that is not significantly slower than ternary-complex interconversion.

Kinetic deductions for a rapid-equilibrium random-order mechanism

Each binary complex dissociation constant ($K_s$) for SAT was derived according to Dalziel and Dickinson [26] 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 is its counterpart ($K_m$) for the ternary complex (Table 1). The data therefore indicate that there is a decrease in the affinity of SAT for each of its substrates during the transition from the binary to the productive ternary complex, a phenomenon observed with chloramphenicol acetyltransferase, another member of the trimeric family of bacterial O-acetyltransferases [12], and described as ‘negative

Figure 2  Hanes plot for SAT utilizing serine as the acyl acceptor and PrCoA as the acyl donor

Top panel: Hanes plot for the acylation of serine by SAT over a range of serine and fixed PrCoA concentrations. The final enzyme (monomer) concentration was 12.2 nM and the incubation conditions were as described in the Materials and methods section (pH 7.5 and 25 °C). ×, 1.25 mM PrCoA; +, 2.5 mM PrCoA; □, 5 mM PrCoA; ○, 10 mM PrCoA. Bottom panels: intercept- and slope-replot data taken from the top panel.

Table 1  Kinetic coefficients, at 25 °C and pH 7.5, for (i) natural substrates, (ii) threonine and (iii) PrCoA

The kinetic coefficients are those in the initial rate equation. $\Phi_A/\Phi_K [K_{AB}]$ is the Michaelis constant ($K_m$) for the acetyl acceptor substrate and $\Phi_B/\Phi_K [K_{BA}]$ is the Michaelis constant ($K_m$) for the acetyl donor substrate. Data are means ± S.D. from at least four separate measurements.

<table>
<thead>
<tr>
<th>Forward reaction</th>
<th>$10^3 \times \Phi_A$ (s$^{-1}$)</th>
<th>$10^2 \times \Phi_A$ (s · mM$^{-1}$)</th>
<th>$10^3 \times \Phi_B$ (s · mM$^{-1}$)</th>
<th>$10^2 \times \Phi_{AB}$ (s · mM$^{-2}$)</th>
<th>$\Phi_B/\Phi_K$ (mM)</th>
<th>$\Phi_A/\Phi_K$ (mM)</th>
<th>$\Phi_{AB}/\Phi_B$ (mM)</th>
<th>$\Phi_{AB}/\Phi_A$ (mM)</th>
<th>$1/\Phi_A$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) AcCoA + serine*</td>
<td>2.4 ± 0.11</td>
<td>2.75 ± 0.18</td>
<td>0.45 ± 0.06</td>
<td>0.335 ± 0.065</td>
<td>1.17</td>
<td>0.2</td>
<td>0.13</td>
<td>0.75</td>
<td>427</td>
</tr>
<tr>
<td>(ii) AcCoA + threonine</td>
<td>2.0 ± 0.7</td>
<td>3800 ± 1600</td>
<td>1.50 ± 0.87</td>
<td>400 ± 170</td>
<td>1900</td>
<td>0.75</td>
<td>0.11</td>
<td>270</td>
<td>500</td>
</tr>
<tr>
<td>(iii) PrCoA + serine</td>
<td>40 ± 32</td>
<td>300 ± 150</td>
<td>40 ± 25</td>
<td>125 ± 23</td>
<td>7.8</td>
<td>1</td>
<td>0.4</td>
<td>3</td>
<td>25</td>
</tr>
</tbody>
</table>

* Data taken from Hindson and Shaw [1].
Serine acetyltransferase specificity and control

Figure 3 Hanes plot for SAT showing mixed non-competitive inhibition by glycine with respect to PrCoA

Top panel: Hanes plot for the acylation of serine by SAT in the presence of a fixed concentration of serine (10 mM) and over a range of PrCoA and fixed glycine concentrations. The final enzyme (monomer) concentration was 30.3 nM and the incubation conditions were as described in the Materials and methods section (pH 7.5 and 25 °C). △, 180 mM glycine; ○, 80 mM glycine; □, 30 mM glycine; ×, 10 mM glycine; ○, 0 mM glycine. Bottom panels: intercept- and slope-replot data taken from the top panel.

co-operativity’ [27]. Interestingly, the decrease in affinity of SAT for serine and PrCoA on proceeding to the ternary complex amounts to a free energy change ($\Delta G^0_c$) of 0.58 kcal·mol$^{-1}$, whereas that for formation of the SAT–AcCoA–threonine ternary complex amounts to 1.2 kcal·mol$^{-1}$, significantly more than that for formation of the SAT–serine–AcCoA ternary complex (0.3 kcal·mol$^{-1}$). Hence the additional methylene groups of PrCoA and threonine must introduce a greater degree of hindrance to ternary-complex formation.

Kinetically derived dissociation constants

It has been shown that 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. A similar treatment can be shown to be applicable to the PrCoA hydrolysis reaction; namely that $k_{+2} \ll k_{-1}$ and hence that $K_m$ reduces to $K_s$ [11].

The $K_m$ for hydrolysis, for PrCoA (370 μM), is in good agreement with the kinetically derived $K_s$ value for propionyl transfer (397 μM), in accordance with the proposed rapid-equilibrium random-order mechanism for propionyl transfer to serine. Moreover, the thioesterase data for PrCoA support the contention that the larger propionyl group compromises binding at the coenzyme-binding site, as shown by the catalytic rate constant ($k_{cat}$) and specificity constant ($k_{cat}/K_m$) for the hydrolysis of PrCoA, which are 17- and 90-fold lower than those for AcCoA hydrolysis respectively [11].

Figure 4 Hanes plot for SAT showing mixed non-competitive inhibition by ATP with respect to serine

Top panel: Hanes plot for the acylation of serine by SAT in the presence of a fixed concentration of PrCoA (0.3 mM) and over a range of serine and fixed ATP concentrations. The final enzyme (monomer) concentration was 48.8 nM and the incubation conditions were as described in the Materials and methods section (pH 7.5 and 25 °C). △, 80 mM ATP; +, 35 mM ATP; □, 15 mM ATP; ×, 5 mM ATP; ○, 0 mM ATP. Bottom panels: intercept- and slope-replot data taken from the top panel.
Figure 5  Hanes plot for SAT showing competitive inhibition between cysteine and AcCoA

Top panel: Hanes plot for the acetylation of serine by SAT in the presence of a fixed concentration of serine (3 mM) and over a range of AcCoA and fixed cysteine concentrations. The final enzyme (monomer) concentration was 12.8 nM and the incubation conditions were as described in the Materials and methods section (pH 7.5 and 25 °C). △, 3 µM cysteine; ×, 2 µM cysteine; ○, 1 µM cysteine; ○, 0 mM cysteine. Bottom panel: intercept-replot data taken from the top panel.

Kinetic studies of cysteine

The results of inhibition studies of the forward reaction were in accord with those of Kredich and co-workers [1,2], namely that cysteine inhibits competitively with respect to AcCoA and mixed non-competitively with respect to serine (Figures 5 and 6). Re-plots of slopes versus [cysteine] for both the competitive and mixed non-competitive primary double-reciprocal plots were, however, parabolic rather than linear, suggesting that either (i) cysteine combines twice with SAT along the reaction sequence and hence resembles serine [11], or (ii) the affinity of SAT for cysteine is effected by its binding at adjacent active sites within the proposed hexamer by an allosteric mechanism. The dissociation constant for cysteine was estimated from a secondary replot of the data to be 0.75 µM, in reasonable agreement with that measured by micro-calorimetry of 0.48 µM (see below).

Micro-calorimetric studies of cysteine

The dissociation constant for cysteine (0.48 µM), determined by micro-calorimetry, is in reasonable agreement with the kinetically derived estimate of 0.75 µM (results not shown). Apparent dissociation constants for cysteine were calculated according to eqn (5), using the micro-calorimetrically derived dissociation constants for cysteine and CoA of 0.48 and 148 µM respectively and a kinetic estimate of the dissociation constant for serine of 0.74 mM [11]. These values were compared with experimentally derived values to test whether cysteine competes with serine or CoA for binding to SAT. For example, the micro-calorimetrically derived apparent dissociation constant of SAT for cysteine (1.4 µM), in the presence of a saturating concentration (2.5 mM) of CoA, is substantially less than that calculated on the basis of direct competition with CoA for binding to SAT (8.5 µM), consistent with the view that cysteine does not bind at the CoA-binding site (Table 2). In contrast, derived apparent dissociation constants for cysteine, measured over three fixed concentrations of serine, are in good agreement with those calculated on the basis of a direct competition with serine for binding to SAT (Table 2). Hence, the micro-calorimetric data support the conclusion that cysteine binds at the serine-binding site and, furthermore, that bound cysteine causes a substantial reduction in the affinity of SAT
Experimental values for apparent dissociation constants \(K_{app}\), heats of binding (\(\Delta H\)) and stoichiometric ratios \(n\) are from deconvolution using non-linear least-squares minimization of the micro-calorimetric data. Calculated apparent dissociation constants were determined according to eqn (5).

<table>
<thead>
<tr>
<th>[Serine] (mM)</th>
<th>Micro-calorimetric (K_{app}) ((\mu M))</th>
<th>Calculated (K_{app}) ((\mu M))</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14000 ± 75</td>
<td>0.48 ± 0.03</td>
<td>1.24 ± 0.0042</td>
</tr>
<tr>
<td>5</td>
<td>11600 ± 110</td>
<td>3.9 ± 0.21</td>
<td>3.7 1.36 ± 0.0033</td>
</tr>
<tr>
<td>15.2</td>
<td>11000 ± 630</td>
<td>15 ± 1.1</td>
<td>10.1 0.07 ± 0.04</td>
</tr>
<tr>
<td>28.5</td>
<td>9500 ± 410</td>
<td>18 ± 2.3</td>
<td>19.1 1.2 ± 0.035</td>
</tr>
</tbody>
</table>

for CoA. This effect is similar to that observed between substrates and denoted as negative co-operativity [11].

**Kinetic studies of serine analogues**

Apparent binding energies \(\Delta G_{app} = RT\ln(K_{app}/K_{A})\) for the carboxyl, amino and hydroxymethyl moieties of serine were determined by kinetic studies with ethanolamine, \(\beta\)-hydroxypropanoic acid and glycine respectively (results not shown) according to Fersht [28]. For example, the apparent binding energy of the hydroxymethyl group of serine was derived from the dissociation constants of serine \(K_{A}\) and glycine \(K_{A}\) to be \(-2.0\) kcal \(\cdot\) mol\(^{-1}\). In a similar fashion, the apparent binding energy of the amino group was calculated to be \(-7.6\) kcal \(\cdot\) mol\(^{-1}\), whereas the carboxyl group of serine made an essential contribution to binding, as shown by the failure of SAT to bind or acetylate ethanolamine.

The apparent binding energy of the \(\beta\)-hydroxygroup of serine was derived from the dissociation constant of alanine to be \(-3.5\) kcal \(\cdot\) mol\(^{-1}\) (results not shown), somewhat less than the hydrogen bond dissociation energy of water (\(-6.4\) kcal \(\cdot\) mol\(^{-1}\)) determined *in vacuo* [29]. Furthermore, such data indicate that the apparent binding energy of the hydroxyl group of serine is greater than that of the entire hydroxymethyl functionality. Hence an unfavourable interaction may be imposed upon the introduction of the methylene group of serine into the ‘hydroxymethyl’ binding region of the serine-binding site.

In conclusion, since each of the functional groups of serine has been shown to contribute to the binding of serine to SAT and, furthermore, since the carboxyl provides an essential interaction, it is not surprising that cysteine, which is isostructural, should interact at the serine-binding site. Furthermore, since the exclusion of cysteine from the serine-binding site would require the hydroxymethyl binding region to sterically occlude the larger thiomethyl moiety of cysteine, it is of note that threonine and O-propionyl-serine, which contain the additional methylene and propionyl moieties respectively, are accommodated. The evidence therefore strongly indicates that the hydroxymethyl-binding region of the serine site is unlikely to provide the obligatory interactions necessary for exclusion of cysteine, namely short-range contacts with the hydroxyl group of serine. Finally, the apparent binding energy of the thiol group of cysteine over the hydroxyl group of serine was estimated from the calorimetrically determined dissociation constant for cysteine (0.48 \(\mu M\)) and the kinetically determined dissociation constant for serine (0.74 mM) to be \(-4.4\) kcal \(\cdot\) mol\(^{-1}\).

**Summary**

The kinetic studies presented above, for SAT from *E. coli*, are consistent with a sequential (ternary complex) mechanism proposed recently [11], rather than a double-displacement one, as proposed by Leu and Cook [8, 10]. The studies on the homologous protein from *S. typhimurium*. In contrast with the proposed steady-state random-order mechanism for acetyl transfer to serine, in which the breakdown of the enzyme–serine complex is partially rate-determining, the propionyl-transfer reaction of SAT was observed to satisfy the requirements of a rapid-equilibrium random-order mechanism, wherein the reduction in \(k_{cat}\) is likely to be due to a slower rate of interconversion of the enzyme–substrate and enzyme–product ternary complexes. Such a finding is in keeping with the view that ternary complex interconversion is not significantly faster than \(k_{cat}\) for acetyl transfer. Acetyl transfer to threonine, on the other hand, conformed to a steady-state random-order mechanism. The alternative substrate data therefore provide further evidence in support of a ternary-complex reaction mechanism for SAT of *E. coli* [11], rather than a double-displacement one, as proposed by Leu and Cook [10] on the basis of experiments with the highly homologous protein from *S. typhimurium*. At present a compelling explanation for such divergent conclusions is not at hand, although inspection of the primary double-reciprocal plot (1/\(v\) versus 1/[AcCoA]), at four concentrations of serine) data of Leu and Cook [10] reveals an imperfect fit for low concentration points, suggesting a degree of convergence more characteristic of a ternary-complex mechanism than a double-displacement one. Furthermore, the authors [8] report a biphasic time course for AcCoA hydrolysis by the SAT of *S. typhimurium*, in which an initial gradient (a ‘burst’) greater than the steady-state rate release is interpreted as evidence for the formation of an acetyl–enzyme intermediate. However, measurement of the gradient of the ‘burst’ reveals it to be roughly two orders of magnitude less than \(k_{cat}\) for acetyl transfer (analysis not shown). Hence the ‘burst’ cannot arise from the formation of an acetyl–enzyme intermediate on the double-displacement reaction pathway they have proposed. In summary the data presented here, as well as the ‘principle of parsimony’ in evolutionary biology, argue that not only SAT from *E. coli*, but also its nearly identical homologue from *S. typhimurium*, catalysate 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.

Kinetic studies with serine analogues and micro-calorimetric data provide strong evidence that cysteine binds at the serine-binding site, whereas steady-state kinetic data have been interpreted as evidence that cysteine binds at the CoA site. Hence binding of cysteine to the serine-binding site of hexameric SAT may induce a conformational change within the active sites of each subunit that gives rise to a substantial reduction in their affinity for AcCoA and the apparent phenomenon of competitive inhibition with respect to AcCoA by steady-state kinetics. Moreover, the proposed mechanism may have evolved to preclude the formation of a productive SAT–cysteine–AcCoA ternary complex, which could give rise to the acetylation of cysteine. This interpretation is supported by the failure to detect acetylation of cysteine by SAT (results not shown). Such an adaptation could have arisen to increase the efficiency with which cysteine negatively regulates the first step in its own synthesis. The crystal structure of the complex of *E. coli* SAT and cysteine (V. E. Pye and P. C. E. Moody, personal communication) is also
consistent with the same binding site for both cysteine and serine. The structure shows cysteine bound in the serine site, adjacent to the CoA pocket; furthermore there is no evidence of an allosteric cysteine site.

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