A Two-step Process Controls the Formation of the Bienzyme Cysteine Synthase Complex

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The regulation of enzyme activity through the transient formation of multiprotein assemblies plays an important role in the control of biosynthetic pathways. One of the first regulatory complexes to be discovered was cysteine synthase (CS), formed by the pyridoxal 5'-phosphate-dependent enzyme O-acetylserine sulphydrylase (OASS) and serine acetyltransferase (SAT). These enzymes are at the branch point of the sulfur, carbon, and nitrogen assimilation pathways. Understanding the mechanism of complex formation helps to clarify the role played by CS in the regulation of sulfur assimilation in bacteria and plants. To this goal, stopped-flow fluorescence spectroscopy was used to characterize the interaction of SAT with OASS, at different temperatures and pH values, and in the presence of the physiological regulators cysteine and bisulfide. Results shed light on the mechanism of complex formation and regulation, so far poorly understood. Cysteine synthase assembly occurs via a two-step mechanism involving rapid formation of an encounter complex between the two enzymes, followed by a slow conformational change. The conformational change likely results from the closure of the active site of OASS upon binding of the SAT C-terminal peptide. Bisulfide, the second substrate and a feedback inhibitor of OASS, stabilizes the CS complex mainly by decreasing the back rate of the isomerization step. Cysteine, the product of the OASS reaction and a SAT inhibitor, slightly affects the kinetics of CS formation leading to destabilization of the complex.

The regulation of protein function is achieved through a large repertoire of mechanisms. In particular, in biosynthetic and catabolic pathways, feedback inhibition accounts for much of the regulation, allowing for fast and specific control of enzyme activities. A less appreciated aspect of the regulation of metabolic pathways is the formation of transient protein complexes. Substrate channeling, achieved by protein-protein interactions between enzymes that catalyze consecutive reactions in metabolic pathways, is a well characterized and efficient mechanism for protecting reactive or labile intermediates, for decreasing the transit time of intermediates, and for withdrawing them from competing reactions (1–5). However, protein interaction networks are also aimed at the fine-tuning of enzyme activity by stabilizing selected conformations. In particular, the role of protein complexes in the regulation of the cysteine biosynthetic pathway in bacteria has been recently investigated (6–17). One example is represented by the transient formation of the cysteine synthase complex (CS) (Scheme 1), involving the specific interaction between the enzymes catalyzing cysteine biosynthesis (Scheme 1), serine acetyltransferase (SAT), the product of cysE, and the pyridoxal 5'-phosphate (PLP)-dependent enzyme, O-acetylserine sulphydrylase-A (OASS-A), the product of cysK. These reactions are at the branch point of the sulfur, carbon, and nitrogen assimilation pathways (Scheme 1) (18) and therefore need to be strictly regulated. In Escherichia coli OASS-A also interacts with ATP sulfurylase (19) and in Bacillus subtilis with the repressor CymR (14). OASS-A is known to interact with SAT to form a very tight ($K_d \approx 1 \text{ nM}$) complex (8). In plants, CS acts as a sensor for the levels of sulfur inside the cell, and in enterobacteria, CS function has not yet been assessed (20). The role of CS is not channeling the product of SAT, O-acetylserine (OAS), to OASS (21, 22), and in fact, OAS freely diffuses out of the complex and spontaneously converts to N-acetylserine (23). Both metabolites play a role in the transcriptional control of the cysteine operon; OAS dissociates the complex between OASS-A and the repressor CymR in B. subtilis (14), whereas N-acetylserine is a known inducer of the cysteine operon, likely via interaction with the regulator CysB (24, 25). A further level of complexity in understanding CS function in enterobacteria deals with the interplay among protein-protein interaction networks and the effects of cysteine, a product of the OASS reaction, and bisulfide, the second OASS substrate, which inhibit the activity of SAT and OASS, respectively.

Although the three-dimensional structure of SAT (Fig. 1A) and OASS-A (Fig. 1B) has been determined from different species, including Haemophilus influenzae (9, 26, 27), E. coli (28, 29), and O. furnaces (30), the mechanism of their assembly remains unknown. The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.
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SCHEME 1. Reductive sulfate assimilation pathway in bacteria and its relations with the nitrogen and carbon assimilation pathways. Enzyme names are shown in boldface with the exception of SAT and OASS that are shown in red. Modulation of enzyme or transcriptional activity is shown with blue arrows; cysteine feedback inhibits the activity of SAT; SAT inhibits OASS activity; N-acetylserine, the product of spontaneous O-N transfer of the acetyl group of OAS, induces the transcription of the cysteine operon. Abbreviations used are as follows: ATPS, ATP sulfurylase; APSK, 3′-phosphoadenosine 5′-phosphosulfate sulfotransferase; NADPH-SR, NADPH-dependent sulfite reductase; PGDH, 3-phosphoglycerate dehydrogenase; PSAT, 3-phosphoserine aminotransferase; PSP, 3-phosphoserine phosphatase; GluDH, glutamate dehydrogenase.

EXPERIMENTAL PROCEDURES

Chemicals and Buffers—All chemicals were purchased from Sigma and were used as received.

Bacterial Strains and Plasmids—The gene coding for *H. influenzae* OASS-A was cloned into pET28a as reported previously (9). The gene coding for *H. influenzae* SAT was cloned in the NdeI/BamHI sites of pET28a vector with retention of the hexahistidine tag and the thrombin cleavage site. Recombinant plasmids were chemically transformed in *E. coli* Tuner™ (DE3) cells (Novagen).

Protein Expression and Purification—OASS and SAT were expressed by a standard fermentation procedure. Briefly, a single colony of transformed *E. coli* cells was inoculated in about 250 ml of LB broth and 30 µg/ml kanamycin and incubated overnight at 37 °C with vigorous shaking. 200 ml of the overnight culture were used to inoculate about 3.5–4 liters of growth medium in a fermentation vessel. Medium consisted of TB broth supplemented with 5 mM MgSO₄, 0.5% glucose, and 30 µg/ml kanamycin (37). During fermentation, carried out at 37 °C with vigorous stirring and aeration with oxygen using filtered compressed air, antifoam (Antifoam 204, Sigma, catalog no. A6426) was added dropwise. The pH was maintained at ~7 using a pH-stat, which drives a peristaltic pump connected to reservoirs of 5 M NaOH and 98% glycerol, respectively. In this way, pH changes serve to control the addition of a carbon source commensurate with bacterial growth (37). Expression was induced by the addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside when the absorbance of the cell culture at 600 nm was about 12. Induction was carried out for about 4 h for OASS and 3 h for SAT or until the pH of the culture started to rise above 7.6. Proteins were purified by immobilized metal ion affinity chromatography using standard procedures. The preparation was treated with thrombin and purified by a further round of immobilized metal ion affinity chromatography. OASS and SAT were stored at −80 °C as concentrated stock solutions in 20 mM Hepes, 50 mM NaCl, pH 7.5, and 50 mM Tris, 50 mM NaCl, 1% glycerol, pH 7.5, respectively.

Activity Assays—Serine acetyltransferase activity was measured spectrophotometrically by coupling the production of CoASH to 5,5′-dithiobis(2-nitrobenzoate) (38). A disulfide exchange reaction gives the mixed disulfide between CoASH and 5-thio-2-nitrobenzoate and free 5-thio-2-nitrobenzoate, which absorbs maximally at 412 nm ($\epsilon_{412} = 14,150$ M$^{-1}$ cm$^{-1}$) (39).
Fluorescence Spectroscopy—Fluorescence measurements were carried out using a FluoroMax-3 fluorometer (Horiba Jobin Yvon, Inc.) equipped with a thermostated cell holder. Emission spectra upon excitation at 412 nm were collected between 425 and 650 nm and were corrected for buffer contribution.

Single Wavelength Stopped-flow Spectroscopy—Kinetic experiments were performed in either 100 mM Hepes buffer, pH 7 or 8, or 100 mM Chex, pH 9. Experiments were carried out under pseudo-first order conditions at either 80 nM SAT trimer or 120 nM OASS dimer. The temperature of the loading syringes and the stopped-flow cell compartment was maintained constant with a circulating water bath.

Single wavelength stopped-flow kinetic experiments were performed using an SX-18MV apparatus (Applied Photophysics) equipped with a 75-watt xenon lamp as a light source and a photomultiplier as a detector. The instrument dead time was 1.56 ms. Kinetic traces were collected upon direct excitation of the cofactor at 412 nm. The emission signal was collected at 90° with respect to the excitation source and filtered below 440 nm by a cutoff filter.

The effect of ionic strength on the kinetics of formation of the CS multienzyme complex was measured. The ionic strength of 100 mM Hepes buffer, pH 7, is 0.017 M. To increase the ionic strength to 0.117 and 0.517 M, Pipes buffer was used while maintaining the pH constant at 7. In addition, the specific effect of chloride was tested using 500 mM NaCl in 100 mM Hepes, pH 7 (ionic strength of 0.517 M).

Data Analysis—Single wavelength kinetic traces were first order and were fitted to Equation 1,

\[
A_t = A_0 + A e^{-\frac{t}{\tau}}
\]

(Eq. 1)

where \(A_t\) and \(A_0\) are the fluorescence emission values at a given time and at zero time, respectively; \(A\) is the total fluorescence change, and \(\tau\) is the relaxation time, such that \(k_{obs}\) is 1/\(\tau\).

Kinetic Model—The CS bienzyme complex contains two dimers of OASS and one hexamer of SAT (8). The equilibrium for formation of the complex can be written as shown in Reaction 1,

\[
\text{REACTION 1}
\]

The overall equilibrium in Reaction 1 can be achieved via several possible mechanisms. Four mechanisms were considered as follows: a simple equilibrium binding, cooperative binding of OASS to SAT, a two-step mechanism with a conformational change in OASS followed by binding, and a two-step mechanism with binding followed by a conformational change. However, the kinetics of formation of the complex is simple. Formation of the complex is first order and depends, in a hyperbolic manner, on the concentration of either SAT (with OASS fixed) or OASS (with SAT fixed). In addition, no lag or burst is observed in the time course, once OASS and SAT are mixed in stopped-flow experiments. As a result, data were analyzed according to the following simple Reaction 2 with the combination of two OASS molecules to give the complex behaving independently.

\[
\text{REACTION 2}
\]

In Reaction 2, \(K_d\) reflects the dissociation constant for the initial interaction complex, whereas \(k_3\) and \(k_4\) are the forward and reverse rate constants for an isomerization to give the final CS bienzyme complex. Formation of the initial interaction complex is rapid, although the isomerization is slow. The dependence of the observed first order rate constant \((k_{obs})\) on the concentration of either OASS or SAT with the other component maintained at a constant concentration adheres to Equation 2,

\[
k_{obs} = k_4 + \frac{k_3}{K_d + A}
\]

(Eq. 2)

where \(A\) represents the concentration of the varied component.

Because of the very low value of the intercept on the \(y\) axis, a large error was associated with \(k_4\) that was thus set to zero during fitting.

In some instances, the dependence of observed kinetic constants could also be fitted to a sigmoid dependence (see Ref. 40), with only small improvements in the quality of fitting. However, the absence of an auto-accelerating behavior in the kinetic traces suggests a note of caution, and data were thus fitted to the simplest model.

The dependence of the equilibrium dissociation constant on temperature was fitted to the van’t Hoff Equation 4,

\[
\ln K = -\frac{\Delta H^0}{R T} + \frac{\Delta S^0}{R}
\]

(Eq. 4)

where \(K\) is \((1/K_d)\), the equilibrium constant for formation of the SAT-OASS complex; \(\Delta H^0\) and \(\Delta S^0\) are standard enthalpy and entropy of binding, respectively; \(T\) is absolute temperature, and \(R\) is the gas constant, 0.00199 kcal/mol K. The temperature dependence of the second order rate constant for formation of CS at limiting concentrations of OASS, and SAT was fitted to the Eyring Equation 5,

\[
\ln \left( \frac{k_3}{K_d} \right) = \frac{1}{T} \ln \left( \frac{k}{\hbar} \right) + \frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R}
\]

(Eq. 5)

In Equation 5, \(k_3/K_d\) is the second order rate constant; \(k\) is Boltzmann’s constant (3.298 × 10^{-23} kcal/K); \(\hbar\) is Planck’s constant (1.589 × 10^{-37} kcal-sec); \(\Delta H^0\) and \(\Delta S^0\) are the activation enthalpy and entropy; and \(T\) and \(R\) are as defined above.
RESULTS

Overexpression of SAT and OASS—Stopped-flow studies of protein-protein interactions require high amounts of highly purified proteins. We were successful in combining a high yield expression system using a pET vector transformed into E. coli Tuner (DE3) cells with an optimized fermentation protocol. About 2 g of 99% pure OASS and about 3 g of 99% pure SAT were purified from 4 liters of fermentation medium.

Stopped-flow Kinetic Studies of Formation of Cysteine Synthase—Binding of SAT to OASS results in an increase in the fluorescence emission of the PLP in OASS (8), likely a result of environmental changes that occur as the α-carboxylate of the C-terminal Ile of SAT occupies the α-carboxylate subsite of OASS (Fig. 1C) (8, 9, 31, 41, 42). Specifically, the formation of a hydrogen bond between Thr⁶⁹ in the active site and the α-carboxylate of the OAS in internal Schiff base linkage to PLP is thought to trigger a transition from an open to a closed form of the enzyme in the S. typhimurium OASS. Similarly, the carboxylate of the C-terminal Ile of SAT is thought to generate the closed form of the enzyme as it mimics the carboxylate of OAS (31). The increase in fluorescence emission at around 500 nm, upon excitation at 412 nm (Fig. 2, inset), was used to follow the formation of the CS multienzyme complex. Time courses were obtained, over a range of protein concentrations, under pseudo-first order conditions, varying the concentration of OASS or SAT while keeping the concentration of the other protein constant. Data were obtained at three different temperatures, 20, 12, and 5 °C (Fig. 2). In all cases, the time courses were pseudo-first order and fitted well to Equation 1. Fig. 3, A and B, show the dependence of the observed rate constants on either OASS or SAT concentration, respectively. At 20 °C, $k_{obs}$ is a hyperbolic function of OASS concentration with SAT maintained at a constant level. A fit of the data to Equation 2 gives $K_d$ and $k_3$ values of 4 ± 1 μM and 99 ± 13 s⁻¹, respectively. Extrapolation of $k_{obs}$ to 0 gives a value within error equal to zero for $k_4$, an indicator that the isomerization step favors the tight complex. The second order rate constant, $k_3/K_d$, for formation of the tight complex is $>10^5$ M⁻¹ s⁻¹. If the experiment is carried out with SAT varied and OASS maintained at a constant concentration, $k_{obs}$ is a linear function of SAT (Fig. 3B). However, the same process, formation of the bi-enzyme complex, is monitored no matter which of the components is maintained fixed and which is varied. Plots appear linear in some cases because the highest concentration of the varied protein is at or below $K_d$, and the equation is not as well conditioned to fit the data. Although values of $K_a$ and $k_3$ cannot be estimated, the slope of the linear function is $k_3/K_d$, and the value estimated from a fit to Equation 3 is, within error, identical to that obtained as a function of OASS concentr-
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The van’t Hoff and Eyring plots of 1/$K_d$ and $k_3$/$k_p$, respectively, are shown in Fig. 3, C and D, respectively. Fitting to Equations 4 and 5 allows an estimation of $\Delta H^0$ and $\Delta S^0$ for formation of the encounter complex and $\Delta H^f$ and $\Delta S^f$ for the rate-limiting step. From Equation 4, $\Delta H^0 = 11.9 \pm 1.6$ kcal/mol and $\Delta S^0 = 0.066 \pm 0.005$ kcal/Kmol. From Equation 5, $\Delta H^f = 13.1 \pm 0.1$ kcal/mol and $\Delta S^f = 0.020 \pm 0.002$ kcal/Kmol.

Effect of Ionic Strength and Chloride Ion on the Kinetics of Formation of Cysteine Synthase—Increasing the ionic strength of 0.017 M, which is the approximate value for 100 mM Hepes, gives a significant decrease in the maximum rate of formation of the complex, from 99 to 35 s$^{-1}$, whereas no apparent effect on $K_d$ is observed (supplemental Fig. S1 and Table 2). Increasing the ionic strength to 0.517 M, however, results in the inability to reach saturation as the concentration of OASS is increased (supplemental Fig. S1); the second order rate constant is decreased from about 10$^7$ at 0.017 M to about 10$^6$ M$^{-1}$ s$^{-1}$. Kinetic parameters are summarized in Table 2.

Chloride inhibits the activity of S. typhimurium OASS upon binding to an allosteric site, which results in a partially closed conformation that is different from that obtained when the external Schiff base of the substrate is formed (30). The $K_i$ for chloride is about 40 mM (43), and 500 mM chloride is thus nearly saturating. The dependence of $k_{obs}$ on the concentration of OASS in the presence of 500 mM chloride is shown in supplemental Fig. S1, and kinetic parameters are summarized in Table 2. The rate does not saturate, and the second order rate constant is very low, about 30 times smaller than that measured at 0.017 M ionic strength.

Effect of Cysteine and Bisulfide on the Kinetics of Formation of Cysteine Synthase—Cysteine is a product of the OASS reaction and binds to the active site generating a Schiff base, competing with SAT binding to form CS, with a $K_d$ of around 20 mM at pH 7. In addition, cysteine is a feedback inhibitor of SAT and binds to the SAT active site resulting in a rearrangement of the C-terminal loop (27). The $K_i$ for SAT-cysteine is 10 $\mu$M at pH 7. With L-cysteine maintained at a concentration of 2 mM at pH 7, SAT is fully saturated, although binding of cysteine to OASS is negligible. In agreement, the emission spectrum of OASS (excitation at 412 nm) in the presence of 2 mM cysteine at pH 7 is not significantly different from the emission spectrum observed in the absence of cysteine (data not shown). The stoichiometric ratio of SAT to OASS in the presence and absence of cysteine was 1.22 and 1.33, respectively, and the intensity of the fluorescence emission at saturation was 1.3–1.5 times higher in the absence of cysteine (data not shown). A 2-fold decrease in $k_3$ was observed in the presence of cysteine, although no significant change in the $K_d$ for the complex was observed (Table 3 and supplemental Fig. S2, A and B).

The CS bienzyme complex is stabilized by bisulfide (21, 44), but the meaning of this effect is poorly understood. The $pK_a$ value for the H$_2$S to SH$^-$ ionization is 7, and experiments were carried out at pH 8 where H$_2$S is more than 90% dissociated to bisulfide (43, 45). Traces in the presence of bisulfide are invariably noisier than those collected in its absence. Blanks collected in the presence of bisulfide appeared noisier with respect to those collected on the buffer, an indication that turbidity due to sulfur precipitation can be responsible for the bad quality of the traces. Kinetic parameters obtained in the presence and absence of bisulfide are shown in Table 3 (supplemental Fig. S2, C and D). A small, less than 2-fold, effect on $k_3$ is observed with no effect on the second order kinetic constant. Similar results were obtained using 100 $\mu$M bisulfide (data not shown).

4 E. Salsi, unpublished results.
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**FIGURE 3.** Observed kinetic constants for the interaction of OASS and SAT as a function of temperature. A and B, dependence of $k_{obs}$ on the concentration of OASS and SAT. Experiments were carried out in 100 mM Hepes, pH 7, varying OASS$_{dimer}$ concentration at a fixed 80 nM SAT$_{trimer}$ concentration (A) and varying SAT concentrations at a fixed 120 nM OASS$_{dimer}$ concentration (B) at 20 °C (circles), 12 °C (squares), and 5 °C (triangles). The solid lines through data points represent the fit to Equations 2 and 3. Fitted parameters are summarized in Table 1. C and D, van't Hoff and Eyring plots. The natural logarithm of $K$ (C) and $K/K_0$ (D) obtained from fitting of dependences shown in A and B were plotted against 1/T to obtain the van't Hoff and Eyring plots, respectively. Fitting to Equations 4 and 5 allows us to calculate the following parameters: $\Delta H^o = 11.9 \pm 1.6$ kcal/mol and a $\Delta S^o$ of 0.066 $\pm$ 0.005 kcal/K/mol, from the van't Hoff plot and $\Delta H^o = 13.1 \pm 0.1$ kcal/mol and $\Delta S^o = 0.020 \pm 0.002$ kcal/K/mol from the Eyring plot.

**TABLE 1**

Kinetic parameters for formation of cysteine synthase

Data were obtained at pH 7. Ionic strength was 0.017 M.

|        | 20 °C | 12 °C | 5 °C |
|--------|-------|-------|------|
| $k_{3}$ (s$^{-1}$) | $99 \pm 13^a$ | $81 \pm 12^a$ | $84 \pm 10^a$ |
| $K_s$ (µM) | $4 \pm 1^b$ | $6 \pm 1^b$ | $12 \pm 3^b$ |
| $k_{3}/K_s$ (M$^{-1}$ s$^{-1}$) | $25 \pm 7^a$ | $14 \pm 3^a$ | $(13 \pm 1)^b$ $(8.7 \pm 0.1)^b (4.5 \pm 0.1)^b$ |

*a Parameters were obtained with OASS varied and SAT fixed. Value in parentheses were calculated from the slope of the dependence of $k_{obs}$ on [OASS].

*b Parameters were obtained with SAT varied and OASS fixed. Values in parentheses were calculated from the slope of the dependence of $k_{obs}$ on [SAT].

**DISCUSSION**

Although CS was one of the first complexes involved in a metabolic pathway to be studied (21), its function in the reductive sulfate assimilation pathway is still debated, and the full network of regulatory complexes that underlies sulfur metabolism started only recently to be unveiled. It seems that the role played by CS in plants and bacteria is, at least to some extent, different. In both kingdoms, CS is at the branching point of sulfur and nitrogen assimilation pathways (Scheme 1) (18), but although bacteria rely on cysteine biosynthesis only when cysteine-starved (absence of cysteine in the growth medium), plants are primary producers and have to face situations such as dark and light cycles and changing temperatures that have a great impact on sulfur assimilation and on the biosynthesis of sulfur-containing biomolecules (11, 46). In plants and bacteria, the product of SAT, i.e. OAS, efficiently dissociates the CS complex, whereas bisulfide stabilizes it (20, 21). Furthermore, OASS activity is decreased in CS (34) down to 5% of the value for the uncomplexed enzyme in *E. coli* and to about 50% in *S. typhimurium* (21). Opposed to the report for CS in *E. coli* (35), plant SAT is activated in CS, mainly as a result of an increase in $k_{cat}/K_{ACoA}$ (15). Studies in *Arabidopsis* have suggested a sulfur-sensing role for CS (20, 47). An abundance of bisulfide stabilizes the complex, activates SAT, and leads to maximal production of cysteine. When bisulfide concentration is low, the accumulation of OAS leads to complex dissociation, to activation of the transcription of the cysteine operon (24), and to reduction of SAT activity until recovery of the sulfate-rich status. Until now, a similar regulatory effect of

pH Dependence of the Kinetic Parameters—There are only small differences in the kinetic parameters calculated at pH 7 and 8, mainly accounted for by an increase in the ionic strength from 0.017 to 0.069 M (supplemental Fig. S3). On the contrary, a larger effect is observed at pH 9 (ionic strength 0.026 M), with an increase in $K_d$ from 4 to 13 µM, and a decrease in $k_3$ from 99 to 55 s$^{-1}$, giving a decrease in the second order rate constant of about 8-fold (Table 4).

Activity of SAT in Cysteine Synthase—Initial rates were measured as a function of either acetyl-CoA or serine for SAT. It is reported in the literature that complex formation significantly affects the catalytic efficiency of *Glycine max* SAT, with an 8-fold increase in $k_{cat}/K_{ACoA}$ (15). In the case of *H. influenzae* SAT, there was no significant change in $K_m$ for either substrate, whether SAT is free or in complex with OASS.
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Table 3: Effect of cysteine and bisulfide on kinetic parameters for formation of cysteine synthase

| pH    | +2 mM cysteine | pH 8 | +1 mM bisulfide |
|-------|----------------|------|-----------------|
| k° (s⁻¹) | 99 ± 13° | 57 ± 14° | 91 ± 15° | 53 ± 11° |
| K° (μM) | 4 ± 1° | 5 ± 1° | 3 ± 1° | 5 ± 2° |
| kₘ/Kₘ (M⁻¹ s⁻¹) | 25 ± 7° | 18 ± 5° | 18 ± 7° | 13 ± 1° |
| Kd (M) | 13 ± 1° | 9 ± 1° | 12.0 ± 0.2° | 13 ± 1° |

* Parameters obtained with OASS varied and SAT fixed.
* Values were multiplied by 10⁻⁶.
* Parameters were obtained with SAT varied and OASS fixed. Values were calculated from the slope of the dependence of kₘ on [SAT].

Table 4: Effect of pH on kinetic parameters for formation of cysteine synthase

| pH    | k° (s⁻¹) | kₘ/Kₘ (M⁻¹ s⁻¹) | Kd (M) |
|-------|----------|-----------------|-------|
| pH 7  | 99 ± 13° | 4 ± 1°          | 13 ± 1° |
| pH 8  | 91 ± 15° | 5 ± 1°          | 13 ± 1° |
| pH 9  | 55 ± 10° | 12 ± 0.2°       | 13 ± 1° |

* Parameters were obtained with OASS varied and SAT fixed.
* Values were multiplied by 10⁻⁶.
* Parameters were obtained with SAT varied and OASS fixed. Values were calculated from the slope of the dependence of kₘ on [SAT].

CS in bacteria has not been reported (11), although a central role of CS is demonstrated by the cysteine auxotroph associated with an alteration of complex formation (48).

In the absence of a three-dimensional structure of CS, pre-steady state studies offer a powerful method to gain insight into the mechanism of complex formation. Furthermore, pre-steady state experiments are collected in solution and thus allow an understanding of the dynamics of biological systems that only on rare occasions can be attained by structural methods. In particular, stopped-flow methods allow a study of complex kinetics and determination of microscopic rate constants for multistep mechanisms. They differ from other pre-steady state methods, such as surface plasmon resonance, in that one can study the effect on the microscopic kinetic constants (i.e. on the single steps of the process monitored) of ligands, ionic strength, pH, temperature, and viscosity. However, stopped-flow investigations can only rarely be applied to bi-enzyme complex formation, due to the lack of an intrinsic spectroscopic probe and the need for high amount of proteins (about 150 mg in this study). In this view, CS is an outstanding system for dynamic investigations, due to the presence of a spectroscopic probe for complex formation and to high yields of recombinant protein attained by our group. It is noteworthy to stress that fluorescent probes, here the intrinsic chromophore PLP, are environment-sensitive and allow monitoring of processes that cannot be followed by absorbance spectroscopy.

In the case of H. influenzae CS, data obtained in these studies adhere to Reaction 2 shown under “Experimental Procedures.” The mechanism allows for the rapid formation of a weak interaction complex, which exhibits a dissociation constant, Kₘ. This is followed by the rate-limiting formation of a tight complex involving a conformational change. The tight complex is formed via the first order rate constant kₘ, and the reverse reaction is determined by the rate constant kₚ. At 20 °C, with OASS varied and SAT fixed at 80 nM, Kₘ is about 4 μM. The value of kₘ is close to zero but can be estimated using a combination of the data discussed above, and the K_d overall was estimated by equilibrium fluorescence studies and values available in the literature. K_d overall is the dissociation constant for the (SAT-OASS)²⁴⁴ complex to SAT and OASS in Reaction 2. The overall K_d value is the product of the Kₘ value for dissociation of SAT-OASS to SAT and OASS and the equilibrium constant for the conformational change in the direction of SAT-OASS, i.e. kₚ/kₚ. A value of 0.15 nm was measured for the K_d overall of the complex in E. coli (35), whereas a value of <2 nm was estimated for CS from H. influenzae (8). Thus, for the value of 0.15 nm, Equations 6 and 7 are achieved,

\[ k_d^{\text{overall}} = \frac{k_4}{k_3} \]  (Eq. 6)

and

\[ k_d = k_3 \left( \frac{k_d^{\text{overall}}}{K_d} \right) \]  (Eq. 7)

A value of 0.004–0.05 s⁻¹ for kₚ would be difficult to see in the experiments carried out in this study. Data suggest the overall equilibrium greatly favors the tight form of the SAT-OASS complex, with a K_d overall of 2.5 × 10⁻⁴ to 2 × 10⁻² for the conformational change in the direction of the tight complex.

In principle, when linear and hyperbolic dependences of k_dobs on ligand concentration are observed, they can arise from an isomerization step preceding binding (49). In the case of CS, a step involving SAT isomerization could be feasible, but the good agreement between microscopic rate constants calculated from linear and hyperbolic dependences (see for example Fig. 3, A and B) using Equations 2 and 3 points to a ligand-induced conformational change. The conclusion that can be drawn from these results is that OASS undergoes an open to closed conformational transition, by a mechanism where binding of the C-terminal carboxylate of SAT to the active site of the enzyme triggers active site closure (31). This result becomes even more remarkable in consideration of the lack of structural data pointing to a conformational transition of OASS following complex formation. In fact, a closed conformation, superimposable with that of S. typhimurium OASS complexed with methionine (31), could not be obtained for any of the OASS-SAT C-terminal peptide complexes solved to date (9, 10, 12). A possible explanation is that the crystallization conditions lock an open structure compatible with peptide binding. However, it is more likely that only whole SAT interacting with OASS is capable of triggering the conformational transition.
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whereas the peptides are not. This observation is confirmed by the fact that in many instances the experimentally determined affinity of OASS for peptides is far lower than that for SAT.

The $K_a$ value for OASS-SAT is temperature-dependent (Fig. 3C), and a fit to the van’t Hoff equation gives $\Delta H^o$ of 11.9 ± 1.6 kcal/mol and a $\Delta S^o$ of 0.066 ± 0.005 kcal/K-mol. From the expression $\Delta G^o = \Delta H^o - T\Delta S^o$ (11.9 kcal/mol - (0.066 kcal/K-mol)(293.15 K)), a value of $-7.4 \pm 1.6$ kcal/mol was obtained, equal within error to the value of $-7.2 \pm 0.9$ kcal/mol calculated using $\Delta G^o = -RT\ln(1/K_a)$. Thus, the formation of the initial interaction complex is entropically driven, given the large value of $\Delta S^o$. Increasing the ionic strength results in the inability to saturate, an indication that the $K_a$ has increased significantly; this is also seen in the 6-fold decrease in $k_{3}/K_a$ as the ionic strength increases from 0.117 to 0.517 M. As expected, the effect of ionic strength on a two-step process is mainly at the expense of the first step, i.e. formation of the encounter complex described by $K_a$ values. Taken together, the van’t Hoff analysis and data at high ionic strength suggest that formation of CS involves a desolvation step that drives the encounter between OASS and SAT, whose interaction is mainly stabilized by electrostatic bonds. This finding supports the results of a recent study on A. thaliana CS where the complex generated by docking simulations is mainly stabilized by electrostatic interactions (36). The knowledge of the solvent conditions that stabilize CS can be exploited for the rational optimization of complex crystallization, an elusive goal so far. A fit of $k_{3}/K_a$ in Table 1 to Equation 5 gives $\Delta H^o$ of 13.2 ± 0.1 kcal/mol and $\Delta S^o$ of 0.020 ± 0.002 kcal/K-mol, giving a value of $7.3 \pm 0.1$ kcal/mol for $\Delta G^o$. One can consider $\ln(k_{3}/K_a)$ consisting of $\ln(k_{3})/(1/K_a)$, i.e. a pre-equilibrium formation of the initial OASS-SAT interaction complex followed by a slower conversion to the tight complex. Corrected values of $\Delta H^o$ and $\Delta S^o$ cannot be accurately calculated because of the large errors, but it is clear that the overall enthalpy and entropy of activation is dominated by the formation of the initial interaction complex.

Not surprisingly, given the role of electrostatic interactions in complex formation, the kinetics of CS assembly is pH-dependent, with a 6-fold decrease of the second order rate constant going from pH 7 to 9 (Table 4 and supplemental Fig. S3). From the limited data available, it is hard to derive conclusions. However, it seems likely that residue(s) external to the OASS active site are involved. In fact, binding of SAT C-terminal penta- and decapetides to OASS is almost pH-independent, with a 3-fold increase in $K_a$ values going from pH 7 to 9. Structural and functional data on OASS of A. thaliana and S. typhimurium suggest a role for conserved basic residues at the active site entrance (6, 50), Lys$^{287}$, His$^{321}$, and Lys$^{223}$ (A. thaliana numbering) are fundamental for the SAT-OASS interaction, and their substitution with Ala completely abolishes binding (6), although no interactions were detected between these residues and the SAT C-terminal octapeptide (10). The stopped-flow experimental setup will allow a detailed study of the effect of such mutations on the kinetics of complex formation, and production of the mutants is currently underway in our laboratory.

The network of protein-protein interactions that regulate sulfate assimilation in bacteria is further complicated by the action of effectors that modulate enzyme activity and conformation. As opposed to other fold type II PLP-dependent enzymes, like tryptophan synthase, that bind cations with regulatory effects (51), no cation-binding site has ever been identified on OASS. On the contrary, a site for chloride has been identified in the crystal structure of S. typhimurium OASS (30). Binding of chloride induces a partially closed conformation that is intermediate between the internal aldimine open and the external Schiff base closed conformations. Because of the high dissociation constant for chloride, the biological significance of its binding to OASS is questionable, whereas it seems plausible that, in vivo, the binding site is occupied by bisulfide, which has roughly the same ionic radius (30). Inhibition of OASS activity by chloride and bisulfide (52–54) further supports the stabilization of a partially inactive conformation. Bisulfide is the product of cysteine desulfuration by intracellular desulfurases and could thus inhibit the buildup of the toxic molecule cysteine above a given concentration (30). Furthermore, a stabilizing effect of bisulfide on CS is reported (21, 44). The effect of chloride and bisulfide on the kinetics of complex formation is different, with chloride giving a 16-fold decrease of the second order kinetic constant $k_{3}/K_a$ and bisulfide giving almost no effect. Even though part of the effect of chloride is due to ionic strength, a 3-fold reduction of $k_{3}/K_a$ is ascribed to chloride binding and to a subsequent decrease in the amount of the initial interaction complex, i.e. a higher $K_a$. Bisulfide gives a 2-fold decrease in the value of $k_{3}$ similar to the approximate 3-fold decrease in $k_{3}/K_a$ observed in the presence of chloride. It seems plausible that the presence of chloride and bisulfide mainly affects $k_{3}$ whose value, however, cannot be determined with accuracy due to large errors associated with extrapolation to zero ligand concentration. In agreement with this observation, surface plasmon resonance experiments revealed that low affinity mutants of E. coli SAT exhibit an invariant value of $k_{3}$, but a larger value of $k_{3}/K_a$ (44). However, previous studies (44) showed that $k_{3}$ values in the presence of bisulfide are of the same order of magnitude as those in its absence. It should be noted, however, that by surface plasmon resonance, only an overall $k_{3}/K_a$ value can be calculated, i.e. in a two-step process $k_{3}/K_a$ is given by $k_{3}/K_a$, and if $k_{3}/K_a < k_{3}$, as is true in the current case, the off rate constant is approximately equal to $k_{3}$. In the case of bisulfide and chloride, binding to OASS is thought to stabilize a partially closed conformation, and an increase in $k_{3}$ is expected, but once the encounter complex is formed, a greater affinity of SAT for OASS should be reflected in a decrease of $k_{3}$, i.e. a more stable closed conformation.

Cysteine is a product of the OASS reaction and binds to the active site of the enzyme, forming the external Schiff base of lanthionine, formed upon elimination of SH$^{-}$ from the cysteine external Schiff base followed by the attack of a second cysteine thiol (55). Cysteine is also a feedback inhibitor of its own synthesis, binding to the active site of SAT and competing with acetyl-CoA and serine (27). The feedback control is the most important regulatory mechanism of the cysteine biosynthetic pathway, excluding regulation of gene expression (24). Because cysteine itself can undergo S-acetylation, resulting in a poor inhibitory effect, the simultaneous binding of cysteine and acetyl-CoA to SAT is prevented by an elegant structural stratagem. The C-terminal tail of SAT binds with residues 241–257
to the cofactor-binding site thus decreasing the affinity of acetyl-CoA. Under the conditions tested here, binding of cysteine to OASS is negligible, and thus any observed effect is due to binding to SAT. As reported in Table 3, there is a 2-fold decrease in $k_{cat}/K_m$ for acetyl-CoA (15). We cannot observe the same effect on H. influenzae SAT, in agreement with data in E. coli (35), and therefore, a different mechanism for complex regulation by effectors seems to be present in bacteria and plants. In plants, CS formation relieves the intramolecular inhibition exerted by the SAT C-terminal sequence on its own activity and results in an increase of $k_{cat}/K_m$ for acetyl-CoA (15). It should be noticed, in fact, that this is suggestive of a shift in the equilibrium toward the dissociated enzymes. In plants, CS formation lowers below a given threshold. It should be kept in mind that the anion-binding site identified on SAT, in agreement with data in E. coli (35), and therefore, a different mechanism for complex regulation by effectors seems to be present in bacteria and plants. In plants, the release of the competitive intrastereic inhibition on SAT in the presence of OASS on the acetyl-CoA site allows for the increase in the activity of SAT in response to increased demand (15). It should be noticed, in fact, that this is suggestive of a shift in the equilibrium toward the dissociated enzymes. In plants, CS formation lowers below a given threshold. It should be kept in mind that the anion-binding site identified on SAT, in agreement with data in E. coli (35), and therefore, a different mechanism for complex regulation by effectors seems to be present in bacteria and plants. In plants, the release of the competitive intrastereic inhibition on SAT in the presence of OASS on the acetyl-CoA site allows for the increase in the activity of SAT in response to increased demand (15).

Our results indicate that modulation by cysteine of CS functional properties is at least negligible. In fact, the concentrations of cysteine tested here (e.g. 2 mM), although higher than those physiologically relevant, exerts only a small, if any, effect on the affinity of SAT for OASS. Cysteine concentration under normal growth conditions in E. coli cells is about 200 $\mu$M and increases to about 1.5 mM in sulfur-starved, cystine-treated cells (56), e.g. in a nonphysiological condition. However, evidence suggests a relevant role for bisulfide. Putting together our results with those of previous studies (21, 30, 44), it seems likely that the anion-binding site identified on S. typhimurium OASS does not have any role in the control of OASS activity in vivo. In fact, the $K_f$ for bisulfide is about 50 $\mu$M, and although a direct measurement of bisulfide concentration has never been carried out, it is hard to think that a concentration of $H_2S$ higher than 100 $\mu$M could ever be found inside bacterial cells (24). This point needs further study, but it is likely that bacteria (differently from plants) use CS as a buffer for fully active OASS that can be quickly freed from the complex when cysteine concentration lowers below a given threshold. It should be kept in mind, in fact, that bisulfide, apart from being produced through the reduction of sulfate, is released from cysteine by the action of desulfurases and signals a sulfur-replete condition that needs OASS inhibition.

Conclusions—The rate-limiting step in the formation of the CS is a conformational change, likely an open to closed transition of OASS, that follows the formation of the initial encounter complex. The isomerization step, with a $K_{eq}$ of about $10^8$–$10^9$ in the direction of the tight complex, is thus the main contribution to the reported high affinity of SAT for OASS. Interestingly, the only competitive modulator of CS is OAS, whereas both cysteine and bisulfide act on protein conformation. In fact, cysteine stabilizes a low affinity conformation of SAT where the C terminus is, at least to some extent, engaged in binding to the acetyl-CoA subsite. On the other side, bisulfide strengthens CS by stabilizing a closed conformation of OASS.

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