# The Selectivity for Cysteine over Serine in Coenzyme A Biosynthesis

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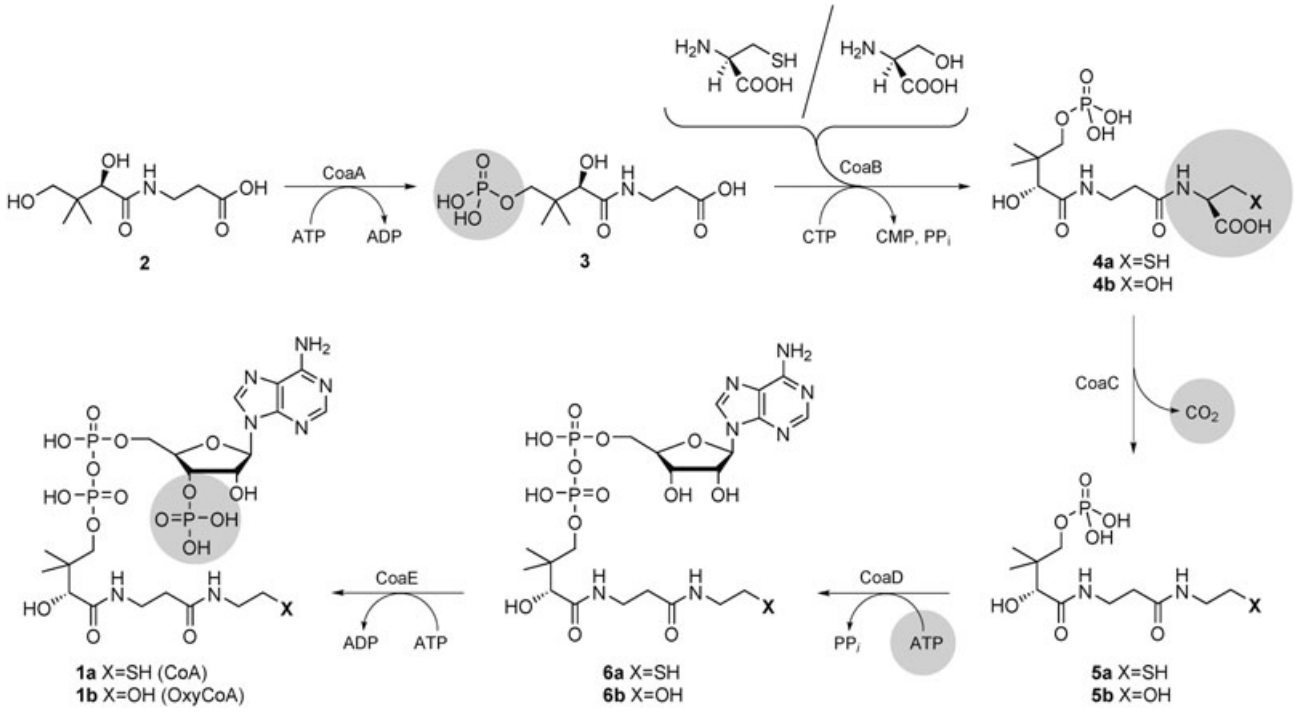
Coenzyme A (CoA, **1a**) is an essential cofactor in all living systems and functions as a carrier and activator of acyl groups in a large variety of enzymatic reactions.[1] In the tricarboxylic acid cycle, acetyl-CoA and succinyl-CoA are key intermediates, while CoA is the source of the 4′-phosphopantetheine cofactor active in fatty acid and polyketide biosynthesis.[2] Non-ribosomal polypeptide synthetases also utilize acyl-group activation by CoA-derived 4′-phosphopantetheine for the formation of peptide bonds,[3] while citrate lyase and malonyl decarboxylase make use of 2′-(5′′-phosphoribosyl)-3′-dephospho-CoA.[4] In each case, the thiol group of either CoA or the CoA-derived cofactor is the essential catalytic functionality; in its absence, or when it is substituted by another functional group, the key thioester intermediate cannot be formed, and CoA is unable to perform its catalytic function. Consistent with this analysis, we have shown that the CoA analogue ethyldethia-CoA, in which the thiol has been replaced with a propyl group, is a potent growth inhibitor of *Escherichia coli*.[5] Furthermore, numerous other biological studies on similar CoA analogues have highlighted these compounds as competitive inhibitors of CoA- and acyl-CoA-utilizing enzymes.[6]

The thiol group of CoA is derived from cysteine, which is selectively incorporated and decarboxylated during CoA biosynthesis. It is critically important that the level of this selectivity is consistently high, since incorporation of other amino acids would lead to the production of potentially toxic CoA analogues. In this communication, we determine the extent of the selectivity exerted by the CoA biosynthetic enzymes for cysteine in the presence of serine (its closest structural analogue), and also identify the two biosynthetic enzymes involved in mediating this selectivity.

CoA is biosynthesized from pantothenate (Vitamin $B_5$) as shown in Scheme 1. We reconstituted this pathway in vitro using the over-expressed biosynthetic enzymes (CoaABCDE)




***Scheme 1.*** *The biosynthesis of Coenzyme A* ***1a*** *and its analogue OxyCoA* ***1b****.*

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*Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.*

from *E. coli*, all of which have been identified.[7] The cysteine/serine selectivity was determined by incubating [$^3$H]-pantothenate ([$^3$H]-**2**, 100 μM) and an excess mixture of L-cysteine and L-serine (200 μM each) with these enzymes followed by the addition of OxyCoA **1b** (~100 μM) as a carrier to facilitate the detection of trace amounts of biosynthesized [$^3$H]-OxyCoA. HPLC analysis of this reaction mixture demonstrated that while [$^3$H]-CoA was readily formed, [$^3$H]-OxyCoA could not be detected (Figure 1). A lower limit for the cysteine/serine selectivity was calculated from the ratio of the radioactive peak area for [$^3$H]-CoA to that estimated for the detection limit for [$^3$H]-

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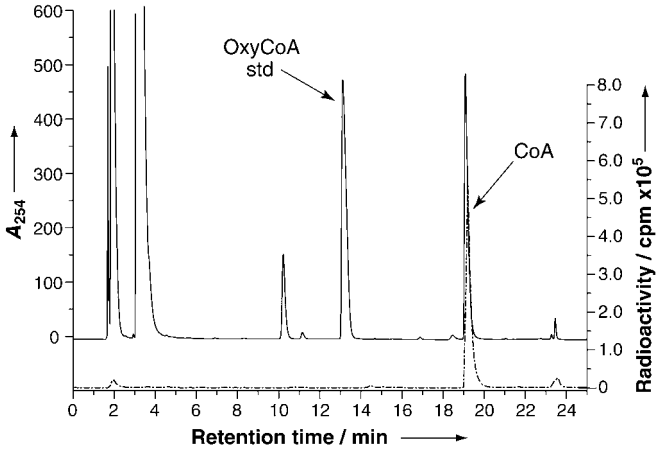


***Figure 1.*** *HPLC analysis of coenzyme A biosynthesis from [$^3$H]-**2** in the presence of equimolar amounts of cysteine and serine with the CoaABCDE enzymes from* E. coli *shows the exclusive incorporation of radioactivity into CoA **1a**. The separation was monitored simultaneously by absorbance detection at 254 nm (upper trace, solid line) and in-line scintillation counting (lower trace, dashed line).*

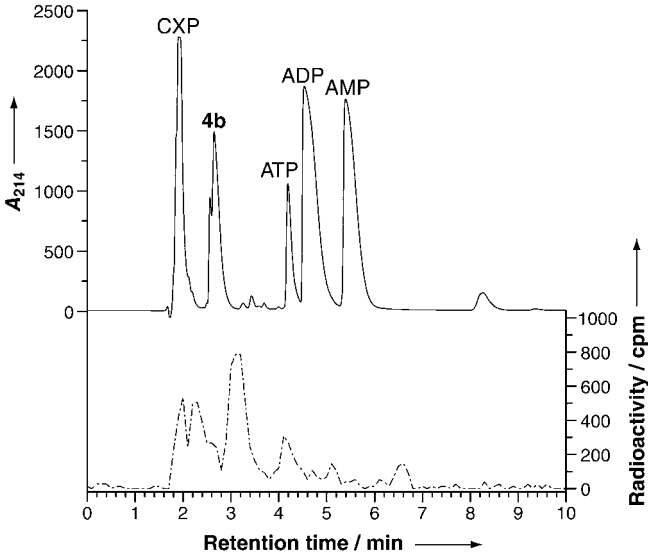


***Figure 2.*** *HPLC traces of reaction mixtures analyzed for possible [$^3$H]-serine incorporation into the oxy-intermediate **4b**. Mixtures were coinjected with authentic **4b** to act as carrier of any small amounts of [$^3$H]-4b that might have been formed. The separation was monitored simultaneously by absorbance detection at 214 nm (upper trace, solid line) and in-line scintillation counting (lower trace, dashed line). Peaks corresponding to cytidine 5′-phosphates (CXP), **4b**, and adenosine 5′-phosphates (AMP, ADP and ATP) are indicated.*

OxyCoA. This gave a selectivity of $>3000$. The experiment was subsequently repeated with a 100-fold excess of serine (2.0 mM) over cysteine (20 μM), and analyzed in the same way. No [$^3$H]-OxyCoA was detected; this demonstrated that the cysteine/serine selectivity ratio is greater than $(5.0 \pm 1.3) \times 10^5$.

We next set out to establish which of the CoaABCDE biosynthetic enzymes are important in determining this high level of selectivity. CoaA can be excluded because it acts on an intermediate prior to the introduction of cysteine (Scheme 1), while CoaD and CoaE can be excluded because both have been shown to exhibit a wide substrate specificity.[6] To probe the selectivity of the bifunctional CoaBC enzyme, unlabeled pantothenate (**2**, 500 μM) was incubated with unlabeled L-cysteine and L-[$^3$H]-serine (200 μM each) in the presence of CoaABCDE, and this was followed by HPLC analysis of the reaction mixture. If CoaB is not cysteine-selective, and all of the selectivity resides with CoaC, the oxy-intermediate **4b** should accumulate. However, even in the presence of authentic **4b** as a carrier, no defined peaks corresponding to [$^3$H]-**4b** could be detected (Figure 2).[8] On the other hand, if selectivity is entirely based on CoaB, any **4b** added to reaction mixtures should be decarboxylated by CoaC to form **5b**. This possibility was tested by incubating **4b** with CoaBC overnight and analyzing the reaction mixture by ESI-MS. No formation of **5b** could be detected in this way (data not shown). These results indicate that both CoaB and CoaC are involved in exerting the large cysteine/serine selectivity found in the biosynthesis of CoA.

The selection of cysteine over serine occurs frequently in biosynthesis, and the mechanistic basis of this selectivity is still poorly understood. Two possible mechanisms have been proposed.[9] The first mechanism is based on selection by noncovalent interactions, such as hydrophobic interactions, the weaker hydrogen bonding of thiols compared to alcohols, and the larger size of sulfur (covalent radius 1.04 Å) compared to oxygen (0.66 Å). Alternatively, cysteine can be selectively bound at the active site by a disulfide bond or by coordination to a zinc ion. It has recently been demonstrated that the cysteinyl-tRNA synthetase from *E. coli*, which is responsible for the selective introduction of cysteine into proteins, utilizes such coordination to zinc to achieve its cysteine/serine selectivity ratio of $10^8$.[10] However, an analysis of the proposed mechanism of formation of **4a** by CoaB suggests a third possible mode for cysteine selection. For this enzyme, amide-bond formation could occur by initial attack of the cysteine thiol on the activated carboxyl of **2a** to give a thioester intermediate, followed by a facile intramolecular S→N acyl shift to give the final product. In such a case, the cysteine selectivity of the enzyme would be based on the greater nucleophilicity of a thiolate compared to an alcohol.

The mechanistic basis for the cysteine/serine selectivity of CoaBC is still incompletely understood. Our current knowledge of CoaB allows us to exclude mechanism two, because the crystal structure of the human enzyme shows the absence of an active site cysteine or zinc ion.[11] Unfortunately, as it has not yet been possible to obtain cocrystals of CoaB with substrate analogues bound at the active site, the relative importance of size effects or thioester formation in mediating the selectivity of this enzyme cannot currently be determined. The first step in the CoaC-catalyzed decarboxylation reaction involves the oxidation of the thiol of **4a** to a thioaldehyde by a tightly bound flavin cofactor.[12] Based on our current knowledge of the mechanism of this enzyme and the crystal structure of the *Arabidopsis thaliana* enzyme with an intermediate bound,[13] we suggest that the selectivity of this enzyme is likely to be due to a combination of size effects and the greater ease of oxidation of a thiol compared to an alcohol moiety.

  

In conclusion, we have demonstrated that the cysteine/serine selectivity in CoA biosynthesis is greater than $(5.0\pm1.3)\times10^5$. This selectivity, mediated by both the CoaB and CoaC enzymes, ensures the production of the active cofactor and avoids the production of the potentially toxic OxyCoA.

## Experimental Section

All chemicals were from Aldrich, Sigma, Acros Organics, or Bachem Bioscience and were of the highest purity. Radiolabeled chemicals were purchased from American Radiolabeled Chemicals (St. Louis, MO). HPLC analyses were performed on a Hewlett–Packard series 1100 HPLC system with in-line scintillation detector (Packard 500TR Flow Scintillation Analyzer with LQTR 300 μL flow cell and Packard Ultima-Flow AP LSC-cocktail) by using a Supelcosil LC-18-T 3 μm, 15 cm×4.6 mm i.d. column (Supelco). CoaA was a generous gift from Suzanne Jackowksi. CoaBC, CoaD, and CoaE were prepared as previously reported.[5] Detailed synthetic procedures for the preparation of OxyCoA **1b** and its precursors are provided in the Supporting Information.

**Overall cysteine selectivity in CoA biosynthesis**: The reaction mixture (100 μL) contained D-2,3-[$^3$H]-pantothenate (0.1 mM, 200 mCi mmol$^{-1}$), ATP (2.0 mM), CTP (1.0 mM), L-cysteine (0.2 mM), L-serine (0.2 mM or 2.0 mM), CoaA (5 μg), CoaBC (5 μg), CoaD (5 μg), and CoaE (5 μg) in Tris-HCl buffer (100 mM, pH 7.6; 1.0 mM dithiothreitol (DTT), 10 mM $MgCl_2$). The reaction was initiated by addition of pantothenate, and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by raising the temperature to 95 °C for 5 min. The precipitated protein was removed by centrifugation (13000 rpm for 5 min). The supernatant (95 μL) was mixed with either water (5 μL) or OxyCoA **1b** (2.5 mM, 5 μL) as carrier. The entire sample was subsequently injected onto the HPLC for analysis, monitoring the separation simultaneously at 254 nm and by in-line scintillation counting.

**Data analysis**: The magnitude of the selectivity was determined by integration of the radioactive peaks and comparison of the peak areas for CoA and OxyCoA. Since no distinct peaks were detected for OxyCoA, a lower value based on an average baseline peak area in the expected elution window (13–14 min) for OxyCoA was used for the calculation.

**CoaB selectivity**: The reaction mixture (100 μL) contained D-pantothenate (0.5 mM), ATP (2.0 mM), CTP (1.0 mM), L-cysteine (0.2 mM), L-[$^3$H]-serine (0.2 mM, 250 mCi mmol$^{-1}$), CoaA (5 μg), CoaBC (5 μg), CoaD (5 μg), and CoaE (5 μg) in Tris-HCl buffer (50 mM, pH 7.6; 1.0 mM DTT, 5 mM $MgCl_2$). The reaction was initiated by addition of the enzymes, and the mixture was incubated at 37 °C for 60 min. The reaction was stopped by raising the temperature to 95 °C for 5 min. The precipitated protein was removed by centrifugation (13000 rpm for 5 min). The supernatant was loaded onto a 500 μL cation exchange column (Dowex 50WX8–100) prepared in a plastic pipette, and the column was washed with deionized water (4×300 μL). The combined eluates were frozen and lyophilized. The resulting residue was dissolved in a buffered solution (50 μL) of **4b** (5.0 mM 4′-phosphopantothenoylserine **4b**, 50 mM Tris-HCl, 5.0 mM $MgCl_2$; pH 7.6). The solution was subsequently injected onto the HPLC for analysis, monitoring the separation simultaneously at 214 nm and by in-line scintillation counting.

**CoaC selectivity**: The reaction mixture (150 μL) contained 4′-phosphopantothenoylserine (5.0 mM) and CoaBC (12.5 μg) buffered in Tris-HCl buffer (50 mM, pH 7.6; 5.0 mM DTT, 5.0 mM $MgCl_2$). The reaction was initiated by addition of the enzyme, and the mixture was incubated at 37 °C for ~16 h. The reaction was stopped by loading the reaction mixture onto a Dowex 50WX8–100 cation exchange column (500 μL) prepared in a plastic pipette. The column was washed with deionized water (4×300 μL), and the combined eluates were frozen and lyophilized. The resulting residue was dissolved in $CH_3CN/H_2O$ (1:1, 200 μL) and analyzed by ESI-MS for the formation of any decarboxylated products.

## Note added in proof

The crystal structure of *E. coli* CoaB has since been determined [S. Stanitzek, M. A. Augustin, R. Huber, T. Kupke, S. Steinbacher, *Structure* **2004**, *12*, 1977–1988] but also fails to unambiguously determine the mechanism of cysteine selectivity. However, the data do suggest that noncovalent interactions of the cysteine thiol with a small hydrophobic cavity might play an important role in selection.

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