Residue Leu-641 of Acetyl-CoA Synthetase is Critical for the Acetylation of Residue Lys-609 by the Protein Acetyltransferase Enzyme of Salmonella enterica*

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Posttranslational regulation of protein function by acetylation is present throughout nature. Regulation of protein function by Sir2 protein (sirtuin) deacetylases is conserved in all domains of life. In the prokaryote Salmonella enterica, the metabolic enzyme acetyl-coenzyme A synthetase (Acs) is regulated by a Sir2-dependent protein acetylation/deacetylation system (SDPADS). The recent identification of the acetyltransferase enzyme responsible for the acetylation of Acs defined the SDPADS in prokaryotes. This report identifies one residue in Acs, Leu-641, which is critical for the acetylation of Acs by the protein acetyltransferase enzyme. In vivo and in vitro evidence shows that mutations at Leu-641 prevent the acetylation of Acs by protein acetyltransferase, maintain the Acs enzyme in its active state, and bypass the need for sirtuin deacetylase activity during growth on acetate.

Sir2 proteins (sirtuins) are biologically conserved NAD\(^+\)-dependent protein deacetylases involved in the posttranslational modification of a wide variety of protein substrates including histones (1–5), tumor suppressor protein p53 (6, 7), and microtubule protein α-tubulin (8). Evidence that archaeal sirtuins regulate gene expression by modulating the acetylation state of the chromatin protein Alba has been reported (9, 10). The interactions of Sir2 family members with their protein substrates have been characterized through structural work (11–16), providing insights into the mechanisms of substrate binding and deacetylation chemistry.

Studies of Gcn5-related N-acetyltransferases (GNATs) identified a superfamily of protein acetyltransferases involved in the acetylation of Sir2 protein targets (17–21). Members of the GNAT superfamily of proteins are widely distributed throughout all domains of life, yet show strikingly limited sequence similarity at the primary amino acid level (21). Factors determining the specificity of these systems in regard to their substrates and molecular partners are only now beginning to emerge (14, 17).

Recent work from our laboratory identified acetyl-coenzyme A (Ac-CoA) synthetase (Acs\(^+\); EC 6.2.1.1) as a substrate of the sirtuin-dependent protein acetylation/deacetylation system (SDPADS) (22). The role of Acs, an enzyme central to the metabolism of all cells, is to activate acetate to Ac-CoA (23). The Acs reaction proceeds via an Ac-AMP intermediate (Fig. 1) before yielding Ac-CoA, a high energy metabolite involved in many anabolic and catabolic processes (23). In Salmonella enterica, Acs activity is posttranslationally regulated via acetylation of residue Lys-609. Acetylation of the latter effectively blocks the conversion of acetate to Ac-AMP (22). Removal of the acetyl moiety from acetylated Acs protein (Acs\(^ac\)) is catalyzed by the S. enterica Sir2 ortholog encoded by the cobB gene (24). As reported earlier, sirtuin-dependent deacetylation of Acs\(^ac\) consumes NAD\(^+\) and restores full activity of the enzyme (22). We recently showed that the pat (formerly yfiQ) gene of S. enterica encodes a member of the GNAT family of acetyltransferase enzymes responsible for the acetylation of Acs (25). The discovery of the protein acetyltransferase (Pat) defined the SDPADS in this bacterium (Fig. 2) (25).

Here we report results from studies aimed at dissecting the molecular interactions between Acs and the S. enterica protein acetyltransferase, Pat. Using genetic selections, we identified a specific residue in Acs, Leu-641, as a critical amino acid for the acetylation of Acs by Pat. We propose that changes at this position alter the protein-protein interactions between Acs and Pat. Residue Leu-641 is hypothesized to be part of the Pat-binding domain of Acs. In vivo and in vitro evidence is presented in support of this conclusion. This work begins defining the mechanism of protein substrate recognition by the SDPADS of S. enterica.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Culture Media, and Growth Conditions**

All bacterial strains used in this study were derivatives of the S. enterica serovar Typhimurium LT2. The genotypes of strains used in this work are listed in Table I. Bacterial strains were grown on no-carbon E minimal medium (NCE) (26), supplemented with potassium acetate as the source of carbon and energy, MgSO\(_4\) (1 mM), and L-methionine (0.5 mM). Luria-Bertani broth (LB) was used as rich medium. Growth behavior was analyzed in 96-well microtiter dishes (BD Biosciences) using a computer-controlled BioTek EL808-I Ultra microplate reader (BioTek Instruments Inc.) with the incubation chamber set at 37 °C. A 2-μl inoculum of an overnight culture of S. enterica was used to seed 198 μl of freshly prepared minimal medium in each well; the medium was supplemented with 10 mM potassium acetate as the carbon and energy source. When used, 0.5 mM arabinose was added to a final concentration of 100 μM. Data points were collected every 10 min, with shaking at the highest intensity for 540 s between readings. All chem...
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Unless otherwise stated, the strains listed were constructed during the course of this work.

<table>
<thead>
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<th>Strain or plasmid</th>
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<sup>a</sup> All S. enterica strains used in this study were derivatives of S. enterica serovar Typhimurium LT2.

<sup>b</sup> In the text, Mud1734 (43) is referred to as MudJ (44).

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**Phage P22 Transductions**—All transductional crosses were performed as described previously (27) using phage P22 HT1051 int210 (28, 29). Transductants were freed of phage as described (30).

**Random Mutagenesis of Acs**—Plasmid pACS1 (acs<sup>-</sup>) was introduced into the mutagenic E. coli strain XL-1 Red (Stratagene) as per the manufacturer’s instructions. The resulting transformants were resuspended into 5 ml of LB medium containing ampicillin and grown overnight at 37 °C with shaking. This culture was subcultured 1:100 (v/v) into 5 ml of fresh ampicillin-containing LB medium and allowed to continue growth at 37 °C with shaking. After the second overnight outgrowth, plasmid was isolated using the Wizard Plus SV Miniprep kit (Promega).

**Isolation of a Plasmid Encoding an Acs Enzyme Insensitive to Acetylation by Pat**—The pool of mutagenized pACS7 plasmid was electroporated (31, 32) into S. enterica strain JE6668 (acs pta cobB), which was unable to grow on any concentration of acetate because of the lack of Acs and phosphotransacetylase (Pta) functions. The cobB mutation ensured that any Acs encoded by the plasmid introduced into the strain would remain acetylated; hence growth on acetate could only occur if the variant Acs protein could escape acetylation by the Pat enzyme. Transformed cells were plated onto NCE minimal agar supplemented with 1 mM MgSO<sub>4</sub>, 0.5 mM l-methionine, 100 mM l-(+)-arabinose (for induction of acs expression), ampicillin, and 10 mM acetate as the sole carbon and energy source. Plates were incubated at 37 °C for 48 h. Clones arising on these plates were recloned into the same solid medium and further characterized. The sequence of the acs gene encoded by plasmids that allowed strain JE6668 to grow on low acetate concentrations was determined using BigDye<sup>TM</sup> protocols (ABI PRISM, University of Wisconsin-Madison Biotechnology Center).

**Construction of Plasmid pACS12 (Acs<sup>L641P</sup>)**—Plasmid pACS10 encoding wild-type Acs protein fused at its C terminus to a chitin-binding domain was used as a template in the PCR-mediated QuikChange® XL site-directed mutagenesis kit (Stratagene). We used mutagenic primers 5’-GGTTGAGAAGCCGTCGAAGAGA-3’ and 5’-CTTCTCTCGAGGCGTTTTCCTCACC-3’ to introduce the L641P mutation (underlined residues identify the mutagenic nucleotide). PCRs were performed as per the manufacturer’s protocol; DNA sequencing confirmed the single base substitution. The resulting plasmid was named pACS12, which carried allele acs<sup>-</sup> that encoded Acs<sup>L641P</sup> protein fused to a chitin-binding domain at its C terminus.

**Construction of Plasmid pACS15 (Acs<sup>L641Q</sup>)**—Plasmid pACS7 carrying the wild-type acs<sup>-</sup> gene under the control of an arabinose-inducible promoter was used as template in the PCR-mediated QuikChange® XL site-directed mutagenesis kit (Stratagene). To introduce the L641P mutation, the mutagenic primers used in the construction of plasmid pACS12 were used. The reactions were performed as per the manufacturer’s protocol. DNA sequencing confirmed the single base substitution. The resulting plasmid was named pACS13, which directed the synthesis of Acs<sup>L641P</sup> protein in response to arabinose in the medium.

**Construction of Plasmid pACS15 (Acs<sup>L641Q</sup>) and Plasmid pACS16 (Acs<sup>L641Q</sup>)**—Plasmid pACS15 and pACS16 were constructed as described for plasmid pACS13, except that mutagenic primers 5’-GGTTGAGAAGCCGTCGAAGAGA-3’ and 5’-CTTCTCTCGAGGCGTTTTCCTCACC-3’ were used.
FIG. 2. Model of posttranslational control of Acs by the SDPADS. Acs proteins are acetylated by the S. enterica Pat in an acetyl-CoA-dependent manner. These acetylated Acs proteins are inactive for the adenylation of acetate (left). CobB, the S. enterica sirtuin deacetylase, Acs in a NAD⁺-dependent manner, releasing 2-O-acetyl-ADP-ribose and nicotinamide. Unacetylated Acs is competent for the conversion of acetate to its adenylated intermediate (acetyl-AMP) and acetyl coenzyme A (right).

FIG. 3. The L641P mutation in Acs bypasses the need for sirtuin deacetylase activity in vivo. A, growth responses of S. enterica strains during growth on acetate (10 mM) as carbon and energy source. Solid squares, strain JE4175 (acs⁺; wild type); solid circles, JE6858 (acs pta cobB/pBAD30 empty vector control); open diamonds, JE7070 + arabinose (acs pta cobB/pAcsL641P); solid diamonds, JE7070 without arabinose (acs pta cobB/pAcsL641P). B, growth on minimal acetate medium with or without the addition of 100 μM L(+)-arabinose for plasmid expression. pAcsWT, pACS7; pAcsL641P, pACS13.

TCTCCACC-3' were used to introduce the L641Q change, and the primers 5'-GTGCTGGAGAAACCGCTCCGAAAGAAG-3' and 5'-CTCTCTCTTCGAGCCCTTCACCAC-3' were used to introduce the L641A change. Plasmid pACS15 directed the synthesis of AcsL641Q protein, whereas pACS16 directed the synthesis of AcsL641A protein, both in response to arabinose in the medium.
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**Biochemical Procedures**

**Purification of Acsc<sup>L641P</sup> Protein**—Acsc protein carrying the L641P mutation was obtained by expressing the *acs* allele carried by plasmid pACS12. The variant protein was purified from *S. enterica cobB* and *cobB* genetic backgrounds as described for wild-type Acsc (22).

**Purification of GST-Pat**—GST-Pat was purified as described (25). Purified GST-Pat protein was dialyzed against 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M KCl and 25% (v/v) glycerol. The protein was drop frozen in liquid nitrogen and stored at −80 °C.

**In Vitro AMP-forming Ac-CoA Synthetase Reactions**—Ac-CoA synthetase activity of Acsc was monitored as described (22, 33, 34). Acsc-dependent synthesis and isolation of radiolabeled Ac-CoA using [14C,C-1]acetate, ATP, and CoA with thin-layer chromatography were performed as described (22), except that 1 μg of unacylated bovine serum albumin was added to each reaction to increase Acsc stability.

**Pat-mediated Acetylation of Acsc**—Protein acetylation reactions using [14C,C-1]acetate, ATP, and CoA with thin-layer chromatography were performed as described (22), except that 1 μg of unacylated bovine serum albumin was added to each reaction to increase Acsc stability.

**Pat-mediated Acetylation of Acsc**—Protein acetylation reactions using [14C,C-1]acetate, ATP, and CoA with thin-layer chromatography were performed as described (22). Briefly, 10 μg of Acsc protein was added to 5 μg of GST-Pat protein in 0.05 M HEPES buffer, pH 7.5, containing 20 μM [14C,C-1]Ac-CoA (specific activity, 47 mCi/mmol) (Moravek) and 200 μM Tris(2-carboxyethyl)phosphine hydrochloride in a final volume of 100 μl. Reactions were incubated for 2 h at 37 °C after which proteins were precipitated by the addition of trichloroacetic acid to a final concentration of 5% (v/v). Precipitated proteins were washed twice with 0.5 ml of ice-cold ethanol and allowed to air-dry. Proteins were resuspended in sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (37) followed by Coomassie staining (36). Polyacrylamide gels were dried onto a piece of Whatman® paper and placed in a sealed film cassette under a Cyclone™ Storage Phosphor Screen (Packard Instruments). Signal intensity was analyzed after 8 h of exposure using the Cyclone™ Phosphor Detection System as per the manufacturer’s instructions.

**Purification of Acs<sub>L641P</sub> Protein**—AcsL641P protein carrying the L641P mutation was obtained by expressing the *acs<sup>L641P</sup>* allele carried by plasmid pACS12. The variant protein was purified from *S. enterica cobB* and *cobB* genetic backgrounds as described for wild-type AcsL641P (22). Purification of AcsL641P protein was dialyzed against 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M KCl and 25% (v/v) glycerol. The protein was drop frozen in liquid nitrogen and stored at −80 °C.

**In Vitro AMP-forming Ac-CoA Synthetase Reactions**—Ac-CoA synthetase activity of AcsL641P protein was monitored as described (22, 33, 34). AcsL641P-dependent synthesis and isolation of radiolabeled Ac-CoA using [14C,C-1]acetate, ATP, and CoA with thin-layer chromatography were performed as described (22), except that 1 μg of unacylated bovine serum albumin was added to each reaction to increase AcsL641P stability.

**Pat-mediated Inhibition of Ac-CoA Synthetase Activity**—AcsL641P protein carrying the L641P mutation was obtained by expressing the *acs<sup>L641P</sup>* allele carried by plasmid pACS12. The variant protein was purified from *S. enterica cobB* and *cobB* genetic backgrounds as described for wild-type AcsL641P (22). Purification of AcsL641P protein was dialyzed against 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M KCl and 25% (v/v) glycerol. The protein was drop frozen in liquid nitrogen and stored at −80 °C.

**In Vitro AMP-forming Ac-CoA Synthetase Reactions**—Ac-CoA synthetase activity of AcsL641P was monitored as described (22, 33, 34). AcsL641P-dependent synthesis and isolation of radiolabeled Ac-CoA using [14C,C-1]acetate, ATP, and CoA with thin-layer chromatography were performed as described (22), except that 1 μg of unacylated bovine serum albumin was added to each reaction to increase AcsL641P stability.

**Pat-mediated Inhibition of Ac-CoA Synthetase Activity**—Acs proteins (Acsc<sup>wt</sup> and Acsc<sup>L641P</sup>) were isolated from *S. enterica* as described above, except that strain JE7462 (pat<sup>cobB</sup>/pat<sup>cobB</sup>Tara) was used as the overexpression host. Acs proteins were acetylated by purified Pat protein in the following manner. A 150-μl total volume reaction containing 0.05 M HEPES buffer, pH 7.5, 200 μM Tris(2-carboxyethyl)phosphine, 40 μM Ac-CoA, and 10% glycerol (v/v) contained either active or heat-inactivated GST-Pat at a concentration of 0.95 μM (18.5 μg); Acs proteins were added to 1.9 μM (20 μg). Reactions were incubated at 37 °C for 2 h. GST-Pat was removed from the reactions by 80 μl of GST-Mag beads (50% slurry (v/v) (Novagen)) pre-equilibrated with the acetyltransferase reaction buffer. Incubation and removal of the magnetic beads was performed as per the manufacturer’s instructions. Reaction supernatants were filtered through 0.45-μm Spin-X<sup>®</sup> centrifuge tube filters (Costar). The protein concentration of the filtered reaction was determined and tested for Ac-CoA synthetase activity as described (33, 34).

**Results and Discussion**

The in vitro and in vivo evidence discussed below indicate that residue Leu-641 of Acsc is critical for the acetylation of the active site residue Lys-609 by the Pat enzyme. Because residue Leu-641 lies close to the site of acetylation in Acsc (Lys-609), we postulate that Leu-641 plays a critical role in the interaction of Acsc with Pat.

**The Variant Acsc<sup>L641P</sup> Enzyme Is No Longer Posttranslationally Modified**—We used a genetic approach to identify derivatives of Acsc that were no longer acetylated by Pat. *cobB* pat<sup>−</sup> strains of *S. enterica* do not grow at low acetate concentrations (<10 mM), because in the absence of CobB sirtuin deacetylase Acsc remains acetylated (i.e. inactive, Fig. 2) (37). The rationale for the search was centered on the premise that if Pat failed to acetylate Acsc in the pat<sup>−</sup> cobB background, Acsc would remain active and cells would grow on acetate despite the lack of CobB sirtuin deacetylase. To isolate variants of Acsc that were no longer acetylated by Pat, we mutagenized plasmid pACSTAcsc<sup>−</sup> (Acsc<sup>−</sup>) and selected for plasmids that would restore growth of the strain JE6668 (acsc<sup>pca</sup> cobB) on minimal medium supplemented with 10 mM acetate as carbon and energy source and L(+)-arabinose (100 μM) as inducer of plasmid-borne *acs*. It was important to inactive the chromosomal copies of *acs* and phosphotransacetylase (pta) to completely block acetyl conversion to A-
CoA (37–39) and eliminate background growth on acetate (Fig. 3B).

Sequencing of the \(\text{acs}\) allele encoded by a plasmid that supported robust growth of strain JE6668 on acetate revealed a single TA to CG transition at nucleotide 1922. This base substitution resulted in a mutant Acs protein with a replacement of a leucyl residue with a prolyl residue at position 641. The L641P mutation was reconstructed using site-directed PCR mutagenesis to confirm the phenotype associated with this mutation. Acs protein encoded by the reconstructed plasmid (pACS13\(\text{acs4}\) (AcsL641P)) restored the ability of strain JE6668 (\(\text{acspta cobB}\)) to grow on acetate when arabinose was added to the medium to induce the synthesis of AcsL641P (Fig. 3A, open diamonds versus solid diamonds). A strain carrying a plasmid encoding wild-type Acs protein failed to grow even when arabinose was included in the medium to induce the synthesis of wild-type Acs protein (Fig. 3B, lower left panel).

To determine whether the substitution of residue Leu-641 by Pro was specific, we replaced Leu-641 with Gln or Ala; the presence of the mutations was verified by DNA sequencing. Growth analyses involving strains carrying plasmid pACS15\(\text{acs5}\) (AcsL641Q) or plasmid pACS16\(\text{acs6}\) (AcsL641A) were performed using a strain carrying plasmid pACS13\(\text{acs4}\) (AcsL641P) as control. Strain JE6668 (\(\text{acspta cobB}\)) harboring the plasmid pACS15\(\text{acs5}\) (AcsL641Q) or pACS16\(\text{acs6}\) (AcsL641A), as well as the strain carrying plasmid pACS13\(\text{acs4}\) (AcsL641P) (data not shown), grew on 10 mM acetate, indicating that the effect of a change on Leu-641 was not specific to the side chain of the prolyl residue and emphasizing the importance of the side chain of the leucyl residue at that position.

**Purified Acs\(^{L641P}\) Is Active in the Absence of CobB-Sirtuin Deacetylase Activity—**Acs\(^{wt}\) and Acs\(^{L641P}\) Acs enzymes were purified from *S. enterica cobB\(^{−}\)* and cobB genetic background to gain insights into the mechanism by which the L641P mutation afforded CobB-independent Acs activity in vivo. Specific activities of these proteins were calculated, measuring the formation of \([14C,C-1]\text{Ac-CoA}\) from \([14C,C-1]\text{acetate}\), Mg(II)/ATP, and HS-CoA (Table II). As expected, Acs\(^{wt}\) purified from the sirtuin-deficient cobB background was substantially less active than the identical enzyme purified from the sirtuin-proficient cobB/H11001 background (36-fold reduction in activity, Table II). Incubation of inactive Acs\(^{wt}\) with CobB sirtuin and NAD\(^+\)/H11001 restored activity to wild-type levels (from 0.1 ± 0.01 to 4.2 ± 0.04 mmol/min/mg protein; Table II). In striking contrast to these results, the Acs\(^{L641P}\) enzyme purified from the cobB background was nearly as active as the Acs\(^{wt}\) enzyme purified from the cobB\(^{−}\) background (3.2 ± 0.09 versus 3.9 ± 0.01 mmol/min/mg protein, respectively). Incubation of CobB and NAD\(^+\) with the Acs\(^{L641P}\) enzyme purified from the sirtuin-deficient background did not change the activity of the Acs\(^{L641P}\) enzyme. In addition, Acs\(^{L641P}\) enzyme isolated from the cobB\(^{−}\) background showed little difference (−1.5-fold) in specific activity when compared with the same enzyme purified from the cobB background (4.7 versus 3.2 mmol of Ac-CoA min\(^{−}\) mg\(^{−}\)).
indicating that the mutant protein was no longer under SDPADS control. These results could be explained by a lack of acetylation of the AcsL641P enzyme by Pat. We also considered the unlikely alternative explanation would be that AcsL641P enzyme gained the ability to activate acetate in the presence of acetylated Lys-609.

**Pat Does Not Acetylate AcsL641P Protein and Does Not Inhibit Its Activity**—To address the possibility that the L641P mutation blocked Acs acetylation by Pat, purified AcsL641P enzyme was incubated with Pat and [14C,C-1]Ac-CoA, and the presence of labeled Acs protein was analyzed by SDS-PAGE and phosphorimaging. Control experiments showed that Pat efficiently transferred the [14C,C-1]acetyl moiety of [14C,C-1]Ac-CoA to wild-type Acs protein (Fig. 4, lanes 2) and that heat-inactivated Pat did not (Fig. 4, lanes 3). These results were consistent with the idea that the AcsL641P enzyme was not acetylated by Pat in vivo, allowing the CobB sirtuin deacetylase-deficient strain, JE6668, to grow on acetate.

To show that purified Pat did not acetylate AcsL641P at Lys-609, we monitored Acs activity after incubation with Pat. As expected, Pat-mediated inhibition of Acs activity was observed (~16-fold reduction). Strikingly, the activity of the AcsL641P enzyme was not significantly affected (10 versus 12 μmol of Ac-CoA/min/mg of protein; Table III). However, the AcsL641P enzyme activity was about one-third that of the AcsL641P enzyme, suggesting that the L641P mutation may affect the stability of the enzyme. The effect of the L641P mutation on Acs activity or stability has not been further characterized.

**Conclusions**—Results from these studies suggest that the structural contributions of residue Leu-641 of Acs are critical for its interaction with the Pat protein acetyltransferase. This conclusion is based on the robust negative effects that changes at position 641 had on the ability of Pat to acetylate Acs, despite drastic changes in the side chain introduced at that position. Although the crystal structures of unacetylated and acetylated Acs from *S. enterica* were solved in the presence of ligands (41), the region of Acs that contains residue Lys-609 was disordered in both models, making it difficult to determine the spatial relationship of Leu-641 to Lys-609. Leu-641 is located in α-helix 20 (residues 637–645), the last α-helix of the Acs protein (Fig. 5), which could suggest that this helix represents a possible binding site on Acs for the Pat protein. Further studies of Pat-Acs interactions are under way.

**REFERENCES**