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E. coli PROPIONYL-CoA SYNTHETASE IS REGULATED *in vitro* BY AN INTRAMOLECULAR DISULFIDE BOND

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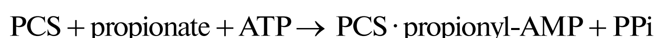
The *E. coli* propionyl-CoA synthetase (PCS) was cloned, expressed, purified, and analyzed. Kinetic analyses suggested that the enzyme preferred propionate as substrate but would also use acetate. The purified, stored protein had relatively low activity but was activated up to about 10-fold by incubation with dithiothreitol (DTT). The enzyme activation by DTT was reversed by diamide. This suggests that the protein contains a regulatory disulfide bond and that the reduction to two sulfhydryl groups activates PCS while the oxidation to a disulfide leads to its inactivation. This idea was tested by sequential mutagenesis of the 9 Cys in the protein to Ala. It was revealed that the C128A and C315A mutants had wildtype enzyme activity but were no longer activated by DTT or inhibited by diamide. The data obtained indicate that two Cys residues could be involved in redox-regulated system through formation of an intramolecular disulfide bridge in PCS.

Propionate can serve as a carbon and energy source in a range of microbes. Propionate oxidation occurs in two steps. First it is activated to propionyl-CoA by the enzyme propionyl-coenzyme A synthetase (PCS, EC 6.2.1.17). Next, the propionyl-CoA is converted to pyruvate by the 2-methylcitric acid cycle [1]. In this variant of the tricarboxylic acid cycle, propionyl-CoA is condensed with oxaloacetate to form 2-methylcitrate. After conversion to 2-methylisocitrate this compound is cleaved to form succinate (which regenerates the oxaloacetate) and pyruvate.

Propionyl-CoA synthetase (PCS) is a member of the adenylate-forming enzyme superfamily all of which catalyze a two step reaction involving an enzyme-bound acyl-adenylate intermediate. In the case of PCS, the two half reactions are:



PCS + propionate + ATP → PCS · propionyl-AMP + PPi

PCS · propionyl-AMP + CoASH → PCS + AMP + + propionyl-CoA

PCS activity in *Salmonella typhimurium* is regulated by a sophisticated mechanism discovered by the Escalante-Semerena laboratory [1–3] based on their earlier work with acetyl-CoA synthetase [4]. PCS is inactivated by propionylation of a Lys residue in active site [5] in a reaction where propionyl-CoA is the propionyl donor. This feedback loop allows high propionyl-CoA levels to down regulate the mechanism for its synthesis. It was found that the enzyme was reactivated by the *Salmonella* sirtuin CobB, which catalyzed the transfer of the propionyl group to NAD^+ to form O-propionyl ADP-ribose thereby liberating the essential Lys residue [5, 6].

During the course of our recent studies on modulating acyl-CoA levels in *E. coli* in order to increase its capacity for producing modified fatty acid synthesis intermediates as a biological source of industrial chemicals we realized that there is a second potential regulatory system for *E. coli* PCS that involved a reversible oxidation and reduction of a disulfide bond within the enzyme. The purpose of this study was to confirm and characterize this potential redox regulation through biochemical studies on the isolated enzyme and to use site-directed mutagenesis to identify the specific amino acid residues involved. We showed that oxidation of the isolated enzyme results in its inactivation while reduction causes the enzyme to be activated. This mechanism is independent of the propionylation mechanism described above and may be involved in further control of enzyme activity.

MATERIALS AND METHODS

Bacterial strains, culture media and growth conditions. All chemicals were purchased from Sigma Chemical Co. (USA) unless otherwise stated. *E. coli* DH5α strain was obtained from New England Biolabs (USA); TOP10 strain and Arctic Express (DE3) were purchased from Invitrogen (USA) and from Stratagene (USA), respectively. *E. coli* strains were cultured at 37°C unless otherwise indicated in Luria-Bertani (LB) broth. Antibiotics ampicillin (100 μg/ml), gentamicin (20 μg/ml) or kanamycin (50 μg/ml) were used in rich medium.

DNA manipulations. Restriction endonucleases, DNA polymerase, and T4 DNA ligase were from Invitrogen Corporation (USA). DNA was sequenced using 3730×l DNA analyzer (Applied Biosystems,

USA). Oligonucleotide primers were synthesized by Integrated DNA Technologies (USA). Unless otherwise stated, DNA manipulations were performed essentially as described by Maniatis et al. [7].

Gene cloning and plasmid constructions. The *E. coli* (k-12) genomic DNA was isolated using the GenElute™ Bacterial Genomic DNA Kit (Sigma, USA). The coding sequence of the PCS enzyme was amplified by PCR with the *E. coli* genomic DNA as a template. The following two oligonucleotide primers were used: (i) a sense primer, 5'-AAGCTTATGTCTTTTAGCGAATTTTA-3' containing a *HindIII* recognition site (underlined) and 20 nucleotides of PCS starting with the ATG start codon; and (ii) an antisense primer, 5'-CTCGAGCTCTTCCATCGCCTGGCGGA-3' containing a *XhoI* recognition site (underlined) and 20 nucleotides that are complementary to the 3'-end sequence of *PCS* without the TAG stop codon. The amplified DNA was subcloned into pGEM T-Easy vector and sequenced. The DNA was digested with *HindIII* and *XhoI* and then inserted into the respective sites of pET-24b to make pET24b-PCS.

Site-directed mutagenesis. Site-directed mutations were made by recombinant PCR [8]. Plasmid pET24b-PCS was used as mutagenesis template. The PCR amplifications were carried out with Platinum™ Taq DNA Polymerase High Fidelity (Invitrogen, USA). The 50 μl PCR reaction used 50 ng of templates, 1 mM primer pair, 200 mM dNTPs, and 2U of DNA polymerase. The extension reaction was initiated by pre-heating the reaction mixture to 94°C for 3 min; 30 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1.5 min; followed by incubation at 72°C for 10 min. The mutagenesis was made by two rounds of PCR. The first round used the sense primer for *PCS* gene with the antisense primer containing the site-directed mutation, and the antisense primer for the *PCS* gene with the sense primer containing the site-directed mutation in two independent PCR reactions. The two PCR products from the first round reactions were purified by the WizardSV Gel and PCR Clean-up System (Promega, USA) after agarose gel electrophoresis. For the second round PCR reaction 1 μl from each of the two PCR products was mixed with 8 μl of dH_2O and 1 μl of the mixture provided the template. Sense and antisense primers for *PCS* were used as primers for the second round PCR. The PCR products were purified as before and cloned into pGEM T-Easy vector and transformed into TOP10 competent *E. coli* cells (Invitrogen, USA). A total of 10 colonies for each mutagenesis were selected and their plasmids were isolated and sequenced. The plasmids with desired mutation were digested with *HindIII* and *XhoI* and then inserted into the respective sites of pET-24b for expression as a His-tagged fusion protein.

Overexpression of PCS in *E. coli* and its purification. For high level protein expression, plasmid pET24b-PCS and the 9 PCS Cys to Ala mutants were electroporated into ArcticExpress (DE3) *E. coli* competent cells (Stratagene, USA). The transformed cells were grown in 10 ml LB medium containing 50 μg/ml kanamycin and 20 μg/ml gentamicin and shaken at 24°C. After overnight cultivation, the cultures were inoculated into 500 ml of the same medium, and shaken at 24°C for 24 h. The cultures were induced by IPTG (final concentration is 0.1 mM) and shaken at 10°C for 24 to 48 h.

The cells were harvested by centrifugation, washed twice in 500 mM potassium phosphate buffer (pH 8.0), and then disrupted by sonication on ice using 6 × 10 s bursts at high intensity with 10 s cooling periods between bursts to prepare a cell-free extract. After centrifugation (3000 × g for 15 min at 4°C) the supernatant was applied to a 2 ml Probond™ Nickel-Chelating Resin (Invitrogen, USA) column. All purification procedures were performed at 0 to 4°C according to the Probond™ instruction manual. The enzyme was eluted from the column with 8 ml of native elution buffer (50 mM NaH_2PO_4, 500 mM NaCl, 250 mM imidazole, pH 8.0) and stored at 4°C. The homogeneity of the purified protein was confirmed by SDS-PAGE.

PCS assay. PCS activity was assayed by measuring the amount of [^{14}C] propionyl-CoA produced from labeled ^{14}C-propionate [9, 10]. The assay mix contained 25 mM Tris-HCl, 50 mM KCl, 0.25 mM CoASH, 2.5 mM [1-^{14}C] K-propionate (4 × 10^6 cpm/μmole), 2.5 mM ATP, 5 mM $MgCl_2$ (pH 8.0) in a total volume of 0.1 ml. The amount of enzyme used ranged from 2.6 to 4.1 μg per assay for the purified enzyme and from 90 to 131 μg per assay for the crude bacterial extract. After 30 min of reaction at 26°C, a 75 μl aliquot of the reaction mix was transferred to a 2.5 cm DE-81 filter disk (Whatman, USA) which was promptly quenched in 2% acetic acid with 100 mM Na propionate. The filter disks were subjected to three 5-min washes in the same solution of acetic acid and Na propionate, rinsed briefly in acetone, and air dried. The amount of filter-bound radiolabeled propionyl-CoA was determined by liquid scintillation counting. Assays were performed in duplicate or triplicate. One unit of activity was defined as 1 μmol of propionyl-CoA produced per minute.

RESULTS

Characterization of recombinant purified PCS. PCS recombinant His-tag fusion protein was affinity purified and characterized by SDS-PAGE analysis. Two bands were observed following Coomassie Blue staining. One band had the expected molecular mass of approximately 70 kDa while the second band, tentatively identified as a PCS degradation product, had a lower molecular weight (Fig. 1). Average overall yield of recombinant protein was estimated at about 2 mg of

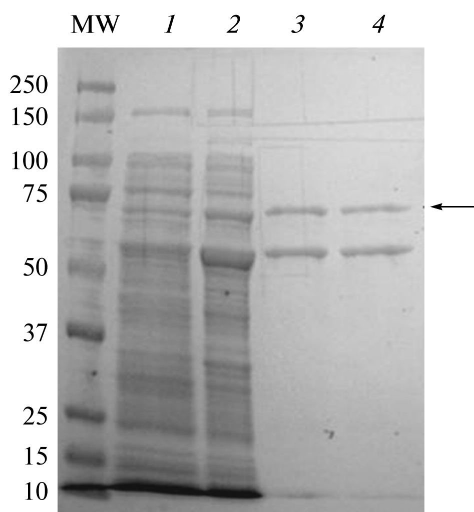


Fig. 1. Expression of *E. coli* PCS analyzed by SDS-PAGE. MW – molecular weight markers; *1* – crude extract from *E. coli* transformed with pET24b; *2* – crude extract from *E. coli* transformed with pET24b-PCS; *3* – crude pET24b-PCS sample purified with a nickel column and run without DTT in SDS loading buffer; *4* – crude pET24b-PCS sample purified with a nickel column and run with DTT in the SDS-PAGE loading buffer. The arrow indicates the expected position of PCS.

fusion protein purified per liter of induced *E. coli* cell culture.

The ability of the expressed PCS to use propionate and acetate as substrates was analyzed. When using propionate as substrate for the enzyme, the V_{max} is 0.43 U/mg, and the K_m is 0.052 mM; when using acetate as substrate, the V_{max} is 0.17 U/mg, and the K_m is 2.13 mM. Attempts were made to use isobutyrat as substrate, but no activity was detected. Clearly propionate is the preferred substrate for the enzyme with little activity toward acetate expected at physiological substrate concentrations.

The effect of redox reagents on the PCS activity. PCS activity was greatly increased by preincubating the enzyme with the reducing agent dithiothreitol (DTT). Maximal enzymatic activity was observed at a DTT concentration of 10 mM (Fig. 2). In the absence of added DTT, the PCS activity was only 10% to 20% of maximum.

The results of DTT and diamide treatments of the *E. coli* PCS enzyme are shown in Fig. 3. Samples of enzyme were preincubated with 10 mM DTT or 10 mM diamide (sometime followed by DTT) for 30 min before the enzyme activity was assayed. The enzyme was activated by DTT and inhibited by diamide in a reversible manner. In our previous work, we found that the *Arabidopsis* acetyl-CoA synthetase also shows the same property, and it was assumed to contain a redox-sensitive thiol group [10]. The similar results with the *E. coli* PCS suggest that it contains a redox active thiol group and that the enzyme is active

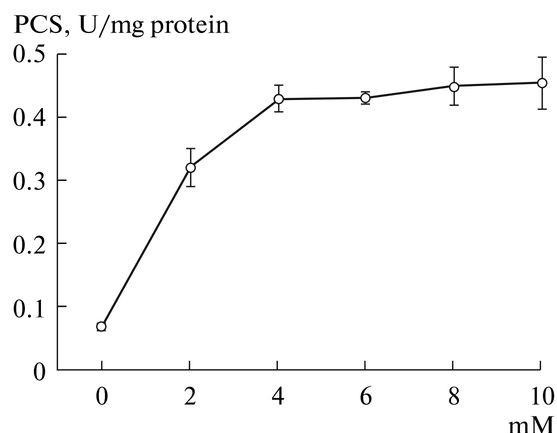


Fig. 2. The effect of different DTT concentrations on the activity of *E. coli* PCS. The purified enzyme was incubated with the indicated DTT concentration for 30 min before the enzyme assay.

when the thiols are reduced to two Cys and inactive when they are oxidized to a cystine residue.

Characterization of the role of Cys residues in PCS. There are 9 Cys in the *E. coli* PCS. To directly test the role of each cysteine in the redox regulation, a series of site specific Cys to Ala mutations were constructed, and the mutant proteins were expressed in *E. coli*. PCS activity was measured in crude extracts of bacterial cells with or without added DTT (Table 1). The wild type and most of the mutant enzymes were activated from 4 to 10-fold by DTT treatment. Activation was always less in crude extracts than in purified proteins suggesting that the Cys residues became more oxidized during the purification process. The two exceptions were C315A and C128A. For these two mutants DTT

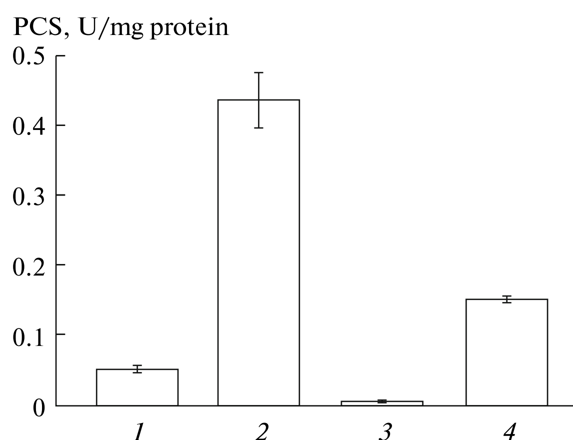


Fig. 3. The effect of DTT and diamide on *E. coli* PCS activity. *1* – PCS preincubated without DTT for 30 min; *2* – PCS preincubated with 10 mM DTT for 30 min; *3* – PCS preincubated with 10 mM diamide for 30 min; *4* – enzyme preincubated with 10 mM diamide for 30 min and then with 10 mM DTT.

Role of Cys in *E. coli* PCS activity and DTT activation of the enzyme. PCS proteins with the indicated Cys to Ala mutants were expressed in *E. coli*. The cells were disrupted and the enzyme activities measured with or without incubation in DTT

Type of PCS	PCS activity + 10 mM DTT, U/mg protein	DTT activation, fold
WT	0.36 ± 0.01	5.0
C47A	0.45 ± 0.03	4.0
C54A	0.50 ± 0.03	9.4
C236A	0.48 ± 0.02	10.3
C279A	0.24 ± 0.01	4.5
C431A	0.41 ± 0.01	4.2
C450A	0.44 ± 0.03	7.8
C602A	0.22 ± 0.01	10.2
C315A	0.83 ± 0.03	1.5
C128A	0.51 ± 0.07	1.5

activation was only about 1.5 fold. In other words, these two mutant enzymes were functionally fully activated without the DTT treatment. Also neither mutant enzyme was inhibited by more than 50% by diamide treatment (data not presented). These results suggest that the disulfide bond(s) that was reduced by DTT treatment and formed by diamide treatment involved Cys 128 and Cys 315.

It is interesting that none of 9 Cys to Ala mutants had activity in the presence of DTT that was less than 60% of the activity of the wildtype enzyme. These data include the Cys 128 and Cys 315 mutants. This suggests that none of these Cys residues is essential for the catalytic function of PCS and that the redox control of the enzyme's activity is not directly caused by regulating the catalytic core of the active site.

Based on the results presented above, the most reasonable explanations for the redox control shown are either an intramolecular disulfide bond formed between C128 and C315 of one protein molecule or bonds involving these two residues on different PCS molecules. These could include intermolecular C128–C315, C128–C128, or C315–C315 bonds (in the latter two cases reduction of both bonds would be necessary for full activation). This question was approached by examining how the oxidized and reduced forms of the proteins migrated in SDS-PAGE. As can be seen in Fig. 1, there is no apparent difference in the migration of the protein following DTT treatment. This indicates that the disulfide bonds were not between two PCS molecules and that C128 and C315 are joined by an intramolecular disulfide bond.

Earlier work has demonstrated that the *Salmonella* PCS is regulated by the reversible propionylation of a Lys residue at the active site of the enzyme [5]. The enzyme CobB catalyzes the removal of the propionate from the active site Lys and the subsequence reactivation of PCS. While this process is mechanistically different from the redox process described here we confirmed that these types of regulation of enzyme activity were independent mechanisms (Fig. 4). With the purified wildtype PCS preparation from *E. coli* used in this experiment both DTT treatment and incubation with recombinant CobB protein and NAD^+ each independently increase the *in vitro* rate of PCS by approximately 5-fold. When DTT and CobB/NAD^+ were added at the same time, PCS activity increased over 25-fold. Clearly, the two mechanisms of regulation of the PCS activity function independently.

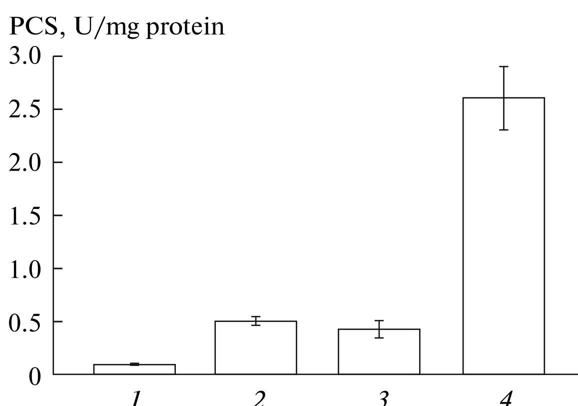


Fig. 4. The activation of the wildtype *E. coli* PCS by DTT and CobB/NAD^+. The enzyme was incubated in the conditions indicated for 30 min before activity was assayed. *1* – control; *2* – +10 mM DTT; *3* – +0.40 μg of CobB and 1 mM NAD^+; *4* – +0.40 μg of CobB, 1 mM NAD^+ and 10 mM DTT.

DISCUSSION

PCS is the first committed step in propionate metabolism and has been shown to be regulated by a feedback inhibition that involves the reversible propionylation of an essential Lys residue [5]. In this report we have identified a potential redox-dependent regulation of this same enzyme. The reversible formation of an intramolecular disulfide bond between C128 and C315 appears to have the potential to control the *E. coli* PCS activity *in vitro*. The formation of the bond, either naturally or following treatment with diamide inhibited the enzyme while reduction with DTT stimulated activity. Mutagenizing either of these Cys residues to Ala resulted in an enzyme that is fully activated irrespective of the redox treatment of the protein.

The *E. coli* PCS appears to undergo redox regulation *in vitro* via an intramolecular sulfhydryl-disulfide mechanism. Does this mechanism contribute to regulating propionate metabolism *in vivo*? Generally it is assumed that structural disulfides in the cytosol of

E. coli are fully reduced and that disulfide oxidation does not occur until the proteins are exported to the periplasm. This process is not the general result of the redox level in the cytosol but is specifically catalyzed by the thioredoxins and glutaredoxin of the thioredoxin and glutathione/glutaredoxin pathways [11]. Not all cytosolic proteins in *E. coli*, however, are reduced with the best known example the OxyR transcription factor containing a disulfide group that can either be oxidized or reduced under physiological conditions [12]. Additional work will be needed in the future to determine of this redox regulation is physiologically significant and if so how it interacts with the propionylation system.

ACKNOWLEDGMENTS

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