Phosphorylation and Formation of Hybrid Enzyme Species Test the "Half of Sites" Reactivity of *Escherichia coli* Succinyl-CoA Synthetase†

Craig J. Mann,‡ Theresa Mitchell, and Jonathan S. Nishimura*

Department of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas 78284

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ABSTRACT: Recent sequencing experiments have identified α-His^{246} as the phosphorylation site of *Escherichia coli* succinyl-CoA synthetase [Buck, D., Spencer, M. E., & Guest, J. R. (1985) *Biochemistry 24*, 6245–6252]. We have replaced α-His^{246} with an asparagine residue using site-directed mutagenesis techniques. The resulting mutant enzyme (designated H246N) exhibited no enzyme activity, as expected, but was found as a structurally intact, stable tetramer. Small differences in the net charge of H246N and wild-type enzymes were first detected on native polyacrylamide gels. These charge differences were resolved by using native isoelectric focusing gels to further separate the wild-type enzyme into diphosphorylated, monophosphorylated, and unphosphorylated species. The enzyme species were found to be interconvertible upon incubation with the appropriate enzyme substrate(s). Sample mixtures containing increasing molar ratios of H246N $(\alpha^{H246N}\beta)_2$ to wild-type enzyme $(\alpha\beta)_2$ were unfolded and then refolded. The refolded enzyme mixtures were analyzed for enzymatic activity and separated on native isoelectric focusing gels. The hybrid enzyme $(\alpha\beta\alpha^{H246N}\beta)$ retained a significant amount of enzyme activity and also exhibited substrate synergism (stimulation of succinate ↔ succinyl-CoA exchange in the presence of ATP). Substrate synergism with this enzyme has been interpreted as evidence for interaction between active sites in such a way that only a single phosphoryl group is covalently attached to the enzyme at a given time [Wolodko, W. T., Brownie, E. R., O'Connor, M. D., & Bridger, W. A. (1983) *J. Biol. Chem. 258*, 14116–14119]. On the contrary, we conclude that tetrameric succinyl-CoA synthetase from *E. coli* is comprised of two independently active dimer molecules associated together to form a "dimer of dimers" that displays substrate synergism within each dimer and not necessarily between dimers. Taken together, the observations described in this work would appear to argue against the hypothesis that *E. coli* succinyl-CoA synthetase is a half of sites reactive protein in which the active sites function in an alternating manner [Bild, G. S., Janson, C. A., & Boyer, P. D. (1980) *J. Biol. Chem. 255*, 8109–8115].

Succinyl-CoA synthetase (SCS)[1] of *Escherichia coli* catalyzes the reaction shown in eq 1 (Nishimura & Grinnell, 1972; Bridger, 1974; Nishimura, 1986). Covalent steps in catalysis

$$\text{succinate} + \text{CoA} + \text{ATP} \leftrightarrow \text{succinyl-CoA} + \text{ADP} + \text{P}_i \quad (1)$$

by the enzyme are described in eqs 2a–c (Nishimura & Grinnell, 1972; Bridger, 1974). The enzyme has an $(\alpha\beta)_2$

$$\text{ATP} + \text{enzyme} \leftrightarrow \text{enzyme-P} + \text{ADP} \quad (2a)$$

$$\text{enzyme-P} + \text{succinate} \leftrightarrow \text{enzyme·succinyl-P} \quad (2b)$$

$$\text{enzyme·succinyl-P} + \text{CoA} \leftrightarrow \text{enzyme} + \text{succinyl-CoA} + \text{P}_i \quad (2c)$$

subunit structure (Bridger, 1971) with two active sites that are located at points of contact between the α- and β-subunits (Bridger, 1974; Vogel & Bridger, 1982; Collier & Nishimura, 1978). The amino acid sequence of both subunits has been deduced from the DNA sequence of the genes (Buck et al., 1985). Available evidence indicates that α-His^{246} is the site of catalytic phosphorylation (Bridger, 1974; Buck et al., 1985).

Published data suggest that the extent of phosphorylation may play a role in the reactivity and stability of this enzyme (Moffet et al., 1972). It has also been suggested that, even though SCS contains two active sites per tetramer, the enzyme exhibits half of sites phosphorylation by ATP, meaning that only one phosphoryl group is present in the enzyme at any one time (Ramaley et al., 1967; Moffet et al., 1972; Bridger, 1974; Bild et al., 1980; Wolodko et al., 1983; Nishimura & Mitchell, 1984a). Substrate synergism, in which partial reactions catalyzed by the enzyme are stimulated dramatically in the presence of a substrate not involved directly in the partial reaction, has been taken as evidence that the two active sites alternate in catalyzing the overall reaction (Bild et al., 1980; Wolodko et al., 1983).

In this study, we have replaced the phosphorylation-site histidine residue with an asparagine residue, producing a completely inactive mutant enzyme designated H246N,[2] as expected. The data show that the wt enzyme can be resolved into three distinct, interconvertible species on the basis of their phosphorylation content. Refolded enzyme mixtures containing H246N and wt were used to demonstrate that the hybrid enzyme species $(\alpha\beta\alpha^{H246N}\beta)$ containing a single competent active site retained a significant amount of activity and exhibited substrate synergism (stimulation of succinate ↔

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* To whom correspondence should be addressed.

‡ Present address: Department of Chemistry, The Pennsylvania State University.

[1] Abbreviations: SCS, succinyl-CoA synthetase; CoA, coenzyme A; SDS, sodium dodecyl sulfate; wt, wild type; kbp, kilobase pairs; IEF, isoelectric focusing; p*I*, isoelectric point.

[2] The single-letter code for amino acids is used to designate the mutant. The first letter denotes the amino acid present in the wild-type enzyme at the numbered position indicated. The final letter denotes the amino acid present in the mutant enzyme at this position.

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succinyl-CoA exchange in the presence of ATP).

Materials and Methods

Bacterial Strains and Plasmids. *E. coli* strain DH5αF′ [φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(r_k^-,m_k^+) *supE44* λ^- *thi-1 gyrA relA1*] was obtained from Bethesda Research Laboratories. Strain CJ236 (*dut1 ung1 thi1 relA1*/pCJ105[Cm^r]) was obtained from International Biotechnologies, Inc. *E. coli* strain TK3D18 (Δ[*kdp-suc*]D18 Δ[*gal-bio*]), which does not express the *sucC* and *sucD* genes (Dr. J. R. Guest, personal communication), was a gift from Dr. Wolfgang Epstein, University of Chicago. Plasmid pGS131 was obtained from Dr. John R. Guest, University of Sheffield. This plasmid contains the *sucC* and *sucD* genes that code for the α- and β-subunits of succinyl-CoA synthetase on a 4.5-kbp *Bgl*II fragment inserted into the *Bam*HI site of pBR322 (Buck et al., 1985). The Bluescript plasmid pSKM was obtained from Stratagene Cloning Systems. Bacteriophage M13mp18 and M13mp19 were obtained from Bethesda Research Laboratories.

Enzymes and Nucleotides. Restriction enzymes were obtained from Bethesda Research Laboratories and New England Biolabs. T4 DNA ligase and T4 polynucleotide kinase were obtained from Bethesda Research Laboratories. Sequenase DNA polymerase was purchased from U.S. Biochemical Corp. Deoxyribonucleotides and dideoxyribonucleotides were purchased from Pharmacia P-L Biochemicals. [^{35}S]dATPαS and [^{35}S]ATPγS were purchased from New England Nuclear.

Oligonucleotides. Oligonucleotides were synthesized by Dr. Robert McGregor, Texas A&M University, and were then purified by gel electrophoresis (Atkinson & Smith, 1984). The following oligonucleotides were prepared: (a) H246N mutagenic oligonucleotide (altered base underlined), dACGTATGGGCAACGCGGGTGC; (b) sequencing primer (110 base pairs upstream from the mutagenic site), dATCGTGATGATCGGTGAG.

Electrophoresis. Restriction enzyme digests of plasmid or phage DNA were analyzed by electrophoresis on agarose submarine gels in Tris–borate–EDTA buffer and visualized by ethidium bromide staining (Maniatis et al., 1982).

Oligonucleotide-Directed Mutagenesis and Expression of SCS Mutant. The procedures used have been recently described (Mann et al., 1989).

Purification of SCS. The wild-type (wt) and mutant enzymes were purified by methods that have been previously described (Grinnell & Nishimura, 1969; Bowman & Nishimura, 1975). Protein concentrations were measured spectrophotometrically with an $E_{280}^{1\%}$ value of 5.11 (Ramaley et al., 1967). SCS activity was assayed by a modification (Grinnell & Nishimura, 1969) of a previously described method (Kaufman, 1955).

Polyacrylamide Gel Electrophoresis. Procedures used for denaturing SDS–polyacrylamide gel electrophoresis were those described by Laemmli (1970). The procedure used for alkaline discontinuous native polyacrylamide gel electrophoresis was described by Blackshear (1984). After electrophoresis, gels were stained with Coomassie Blue R-250 to visualize protein bands.

Isoelectric Focusing (IEF). The method of Robertson et al. (1987) was used for vertical IEF gels using a protein II minigel electrophoresis system (Bio-Rad). For pH 5–7 native IEF gels, the procedures were used essentially as described, with the exception that electrophoresis was performed at 200 V (constant voltage) overnight at 4 °C. The procedures used for pH 5–7 urea–IEF gels were also essentially as described, with the exception that the upper chamber was filled with 50 mM NaOH, the lower chamber was filled with 25 mM H_3PO_4, and electrophoresis was performed at 200 V (constant voltage) overnight at 4 °C. The intensities of protein bands stained with Coomassie Blue R-250 were quantitated by using a Bio Image Visage 110 gel scanner.

Two-Dimensional Electrophoresis. For second-dimension SDS gel analysis, the lane of interest was excised from a native IEF gel (described above), equilibrated with SDS sample buffer [125 μM Tris-HCl, pH 6.8, 20% glycerol, 4% β-mercaptoethanol, 4% SDS (Laemmli, 1970)], and placed in direct contact with the stacking gel of an SDS gel described by Laemmli (1970). For second-dimension urea–IEF gel analysis, the lane of interest was excised from a native IEF gel, equilibrated with lysis buffer [8 M urea, 2% Triton X-100, 1% β-mercaptoethanol (Robertson et al., 1987)], and placed in direct contact with a pH 5–7 urea–IEF gel. Electrophoresis was performed as described above for urea–IEF gels.

Phosphorylation/Dephosphorylation Reactions. The method of Bowman and Nishimura (1975) was used to dephosphorylate and phosphorylate purified wt enzyme samples.

Substrate Interconversion of Enzyme Species. Wild-type enzyme was labeled with [γ-^{32}P]ATP for 15 min at 37 °C. The labeled protein was separated from free label by passage through a Sephadex G-50 column. After concentration of enzyme-containing fractions, the final protein concentration was determined by using purified SCS as a standard (Lowry et al., 1951). Labeled enzyme (10 μg) was incubated with 338 μM CoA and increasing concentrations of sodium succinate (0–25 μM) for 15 min at 37 °C. The samples were then analyzed on native IEF gels as described above.

Hybrid Enzyme Formation. Wild-type and H246N mutant enzymes were mixed together at increasing molar ratios of H246N to wt, while at the same time maintaining a constant amount of total protein (200 μg). Samples containing the enzyme mixtures (5 mg/mL) were denatured in 6 M urea, 5% glacial acetic acid, 0.1 mM NaEDTA, and 1 mM DTT overnight at 4 °C. The mixtures were then diluted to a final protein concentration of 0.15 mg/mL according to refolding conditions described by Pearson and Bridger (1975) in the presence or absence of 400 μM ATP. Refolded mixtures were then assayed for SCS activity (Ramaley et al., 1967) and analyzed on native IEF gels as described above.

Succinate Exchange. Exchange rates of [^{14}C]succinate and succinyl-CoA catalyzed by enzyme mixtures refolded in the absence of ATP were assayed by the method previously described (Nishimura & Mitchell, 1984a).

Results

The replacement of the active-site histidine residue of *E. coli* succinyl-CoA synthetase with an asparagine residue was expected to result in the formation of an inactive mutant enzyme. That this was the case was found upon measurement of the enzymatic activity of the purified H246N mutant protein. This mutant enzyme had essentially no enzyme activity compared to wt enzyme. The migration of H246N and wt enzymes on denaturing and native polyacrylamide gels is shown in Figure 1. Under denaturing gel conditions, wt and H246N exhibited identical behavior (see Figure 1A). The faint band corresponding to approximately 50 kDa has been found only in recombinant protein preparations. Under native conditions (Figure 1B), wt migrated toward the anode slightly faster than H246N. This was likely due to a greater net negative charge of wt enzyme compared to H246N.

Separation of Enzyme Species. The basis for the charge differences was examined by subjecting enzyme samples to

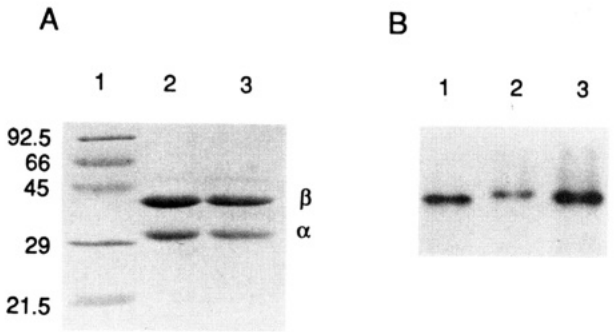

FIGURE 1: Denaturing and nondenaturing polyacrylamide gel electrophoretograms of wt and H246N enzymes. Panel A, samples were applied to a 12% SDS–polyacrylamide gel (Laemmli, 1970): lane 1, molecular weight markers with molecular mass in kilodaltons (92.5, 66, 45, 29, 21.5); lane 2, 10 μg of wt enzyme; lane 3, 10 μg of H246N mutant enzyme. Panel B, samples were applied to a 7.5% native polyacrylamide gel (Blackshear, 1984): lane 1, 10 μg of wt enzyme; lane 2, 10 μg of H246N mutant enzyme; lane 3, 10 μg of wt + 10 μg of H246N.

further analysis on IEF vertical slab gels under native and denaturing conditions. As shown in Figure 2A, wt enzyme migrated as three bands on native IEF gels (lane 2) with isoelectric points (p*I*) of 5.75, 5.35, and 5.05, respectively, with a majority of the protein present in the two lower bands. In contrast, the H246N mutant enzyme migrated as a single major band (lane 3) with a p*I* of 5.58. As will be demonstrated in Figures 3 and 4, the three bands present in wt samples are the unphosphorylated, monophosphorylated, and diphosphorylated forms of this enzyme. Two-dimensional gel electrophoresis was also performed on these samples with native IEF gels in the first dimension followed by SDS or urea–IEF gels in the second dimension. In this experiment (Figure 2B), each of the three bands obtained from wt enzyme samples on native IEF gels contained the β- and α-subunits of SCS. The contaminant band alluded to in Figure 1 migrated between the two more acidic isoelectric species. Additional analysis of a wt sample on a second-dimension urea–IEF gel gave the results shown in Figure 2C. The unphosphorylated species (upper band on first-dimension native IEF gel) separated into one α- and one β-subunit spot. The monophosphorylated species (middle band) separated into one β-subunit spot and two α-subunit spots corresponding to the unphosphorylated and phosphorylated α-subunits present in the monophosphorylated enzyme. The diphosphorylated species (lower band) also separated into one β-subunit spot and one major phosphorylated α-subunit spot. A small amount of unphosphorylated α-subunit appeared as a faint spot that may have come from partial dephosphorylation of the phosphorylated α-subunit during preparation of the sample lane for the second-dimension gel electrophoresis. Subunit spots were assigned on the basis of the migration of purified α-subunit and wt enzyme samples used as standards shown in lanes 1 and 2 of Figure 2C. In contrast, analysis of H246N by second-dimension SDS or urea–IEF gels (Figure 2, panels D and E) produced only one spot for each subunit on each type of gel with a noticeable absence of the phosphorylated α-subunit spot on the second-dimension urea–IEF gel.

Interconversion of Enzyme Species by Substrates. Samples of wt enzyme were next incubated by using conditions that favored the dephosphorylation or phosphorylation of the protein (Figure 3). The enzyme sample that had been incu-

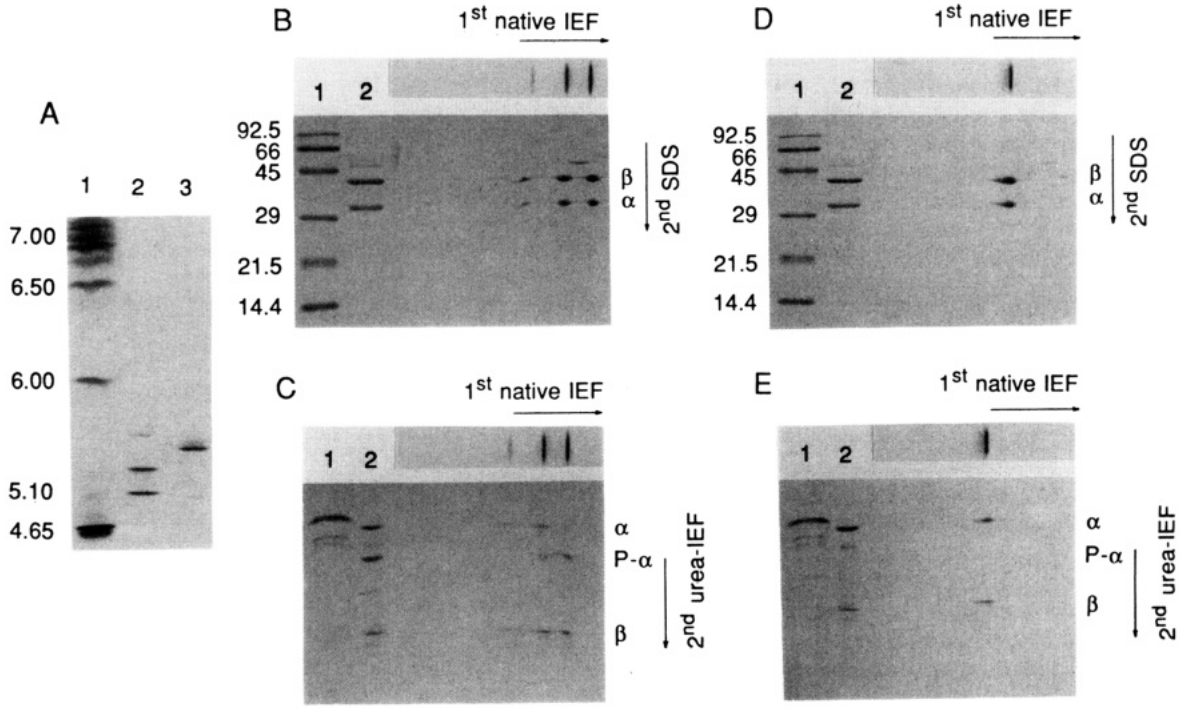

FIGURE 2: Isoelectric focusing and two-dimensional gel electrophoretograms of wt and H246N enzymes. Panel A, samples were applied to a pH 5–7 native isoelectric focusing (IEF) gel (see Materials and Methods): lane 1, IEF standards (Bio-Rad) with isoelectric points in pH units (7.00, 6.50, 6.00,. 5.10, 4.65); lane 2, 10 μg of wt enzyme; lane 3, 10 μg of H246N mutant enzyme. Panel B, a duplicate lane of lane 2 (panel A) containing 10 μg of wt enzyme was excised from the native IEF gel and used for second-dimension SDS gel electrophoresis: lane 1, molecular weight markers with molecular mass in kilodaltons (92.5, 66, 45, 29, 21.5, 14.4); lane 2, 10 μg of wt enzyme. Panel C, a duplicate lane of lane 2 (panel A) containing 10 μg of wt enzyme was excised from the native IEF gel and used for second-dimension pH 5–7 urea–IEF gel electrophoresis (see Materials and Methods): lane 1, 10 μg of purified wt α-subunit; alne 2, 10 μg of wt enzyme. Panel D, a duplicate lane of lane 3 (panel A) containing 10 μg of H246N mutant enzyme was excised from the native IEF gel and used for second-dimension SDS gel electrophoresis: lane 1, molecular weight markers with molecular mass in kilodaltons (92.5, 66, 45, 29, 21.5, 14.4); lane 2, 10 μg of H246N mutant enzyme. Panel E, a duplicate lane of lane 3 (panel A) containing 10 μg of H246N mutant enzyme was excised from the native IEF gel and used for second-dimension pH 5–7 urea–IEF gel electrophoresis (see Materials and Methods): lane 1, 10 μg of purified wt α-subunit; lane 2, 10 μg of H246N mutant enzyme.

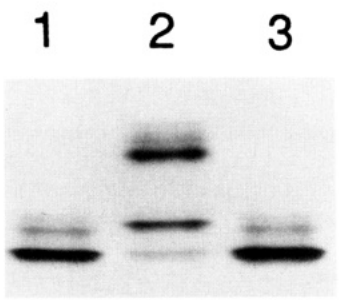

FIGURE 3: Native isoelectric focusing gel electrophoretogram of dephosphorylated or phosphorylated wt enzyme. Samples were applied to a pH 5–7 native isoelectric focusing (IEF) gel (see Materials and Methods): lane 1, 10 μg of wt enzyme as isolated; lane 2, 10 μg of dephosphorylated wt enzyme (see Materials and Methods); lane 3, 10 μg of dephosphorylated wt enzyme rephosphorylated with 4 mM [γ-^{32}P]ATP (see Materials and Methods).

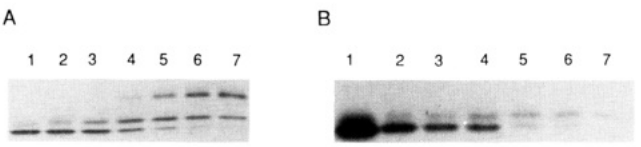

FIGURE 4: Incubation of diphosphorylated wt enzyme ([^{32}P]SCS) with substrates. Aliquots of diphosphorylated wt enzyme (10 μg) were incubated for 15 min at 37 °C with 338 μM CoA and increasing concentrations of succinate (lanes 2–7). Panel A, samples were applied to a pH 5–7 native isoelectric focusing (IEF) gel (see Materials and Methods): lane 1, [^{32}P]SCS; lane 2, succ = 0 μM; lane 3, succ = 0.5 μM; lane 4, succ = 1.0 μM; lane 5, succ = 3.5 μM; lane 6, succ = 7.0 μM; lane 7, succ = 25 μM. Panel B, this autoradiogram was obtained after exposing X-ray film with a duplicate gel of that shown in panel A loaded with identical 10-μg samples: lane 1, [^{32}P]SCS; lane 2, succ – 0 μM; lane 3, succ = 0.5 μM; lane 4, succ = 1.0 μM; lane 5, succ = 3.5 μM; lane 6, succ = 7.0 μM; lane 7, succ = 25 μM.

bated with excess succinate and CoA to dephosphorylate the protein produced the intermediate and slower migrating bands, with a majority of the protein in the slower migrating band (lane 2, Figure 3). Dephosphorylated enzyme subsequently incubated with [γ-^{32}P]ATP incorporated 1.8 mol of P_i/mol of SCS and migrated as the faster moving band (lane 3), thus demonstrating that this band corresponded to a diphosphorylated SCS species. That some of the latter remained after incubation with succinate and CoA indicates the presence of inactive enzyme.

Incubations of phosphorylated wt enzyme ([^{32}P]SCS) with excess CoA and increasing amounts of succinate demonstrated that the middle and upper bands of the three bands present on native IEF gels were indeed the monophosphorylated and dephosphorylated enzyme species, respectively (Figure 4A). Band intensities on the accompanying autoradiogram of an identical gel (Figure 4B) revealed that the amount of the diphosphorylated species (lower band) steadily declined as the succinate concentration increased. The monophosphorylated species (middle band) increased initially and then gradually decreased at higher succinate concentrations, while the dephosphorylated species (upper band) increased as the succinate concentration was increased, as indicated in the Coomassie Blue stained gel (Figure 4A). The apparent loss of ^{32}P from the enzyme forms upon incubation with CoA alone (Figure 4B, lane 2) can be ascribed to CoA-stimulated exchange of ^{32}P enzyme with trace amounts of nonradioactive phosphate (Ramaley et al., 1967) present in the incubaton solution. That deposphorylation was not involved is indicated by the fact that unphosphorylated enzyme was not formed (Figure 4A, lane 2).

Hybrid Enzyme Formation. In these experiments, the inactive H246N mutant enzyme was mixed with active wt enzyme at increasing molar ratios to produce hybrid tetramers ($\alpha\beta\alpha^{H246N}\beta$) containing only one viable active site per tetramer, as illustrated schematically in Figure 5. Enzyme mixtures were refolded in the presence or absence of ATP and then analyzed on native IEF gels. Samples were also assayed for enzyme activity.

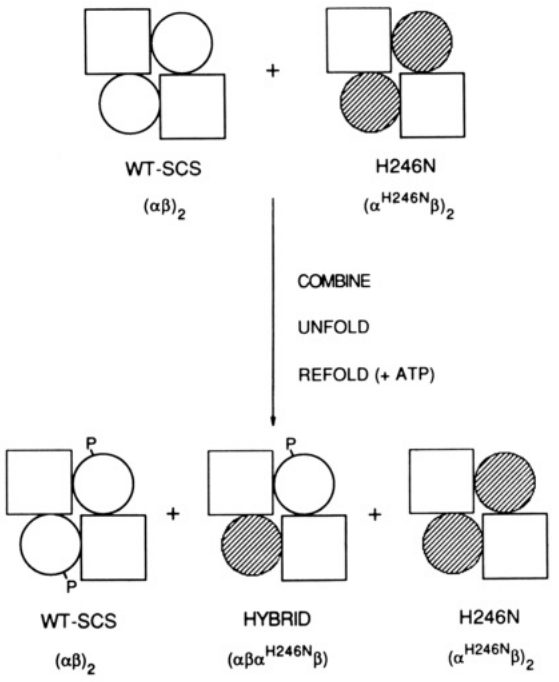

FIGURE 5: Schematic illustration of the formation of hybrid tetramers ($\alpha\beta\alpha^{H246N}\beta$). H246N mutant enzyme and wt enzyme were combined at increasing molar ratios of H246N to wt. Samples were unfolded in 6 M acid/urea overnight at 4 °C and refolded in the presence or absence of ATP. Samples of each mixture were assayed for SCS activity and loaded onto a native IEF gel for separation of tetrameric species.

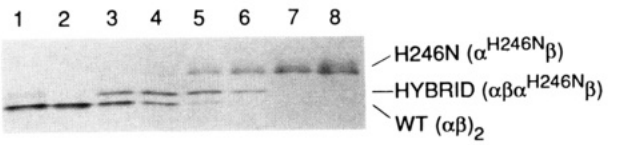

FIGURE 6: Native isoelectric focusing gel electrophoretogram of enzyme mixtures refolded in the presence of ATP. Samples (10 μg) were applied to a pH 5–7 native isoelectric focusing (IEF) gel (see Materials and Methods): lane 1, wt enzyme as isolated; lane 2, refolded wt enzyme; lane 3, 1:1 (mutant:wt) enzyme molar ratio; lane 4, 2:1 enzyme molar ratio; lane 5, 5:1 enzyme molar ratio; lane 6, 10:1 enzyme molar ratio; lane 7, refolded H246N enzyme; lane 8, H246N mutant enzyme as isolated.

Enzyme mixtures refolded in the presence of ATP were separated into wt, hybrid, and H246N tetramers by using native IEF gels (Figure 6). Quantitation of the three different species in each mixture revealed that the amount of wt tetramer present was slightly higher than the calculated value for the anticipated formation of wt tetramer, assuming random assembly of tetramers, in each mixture (Figure 7A). Conversely, the formation of hybrid (Figure 7B) and the formation of H245N tetramers (Figure 7C) were both slightly lower than the calculated values of formation of these tetrameric species in each mixture.

As shown in Table I, experimental activity data, obtained in the presence or absence of ATP during refolding, were

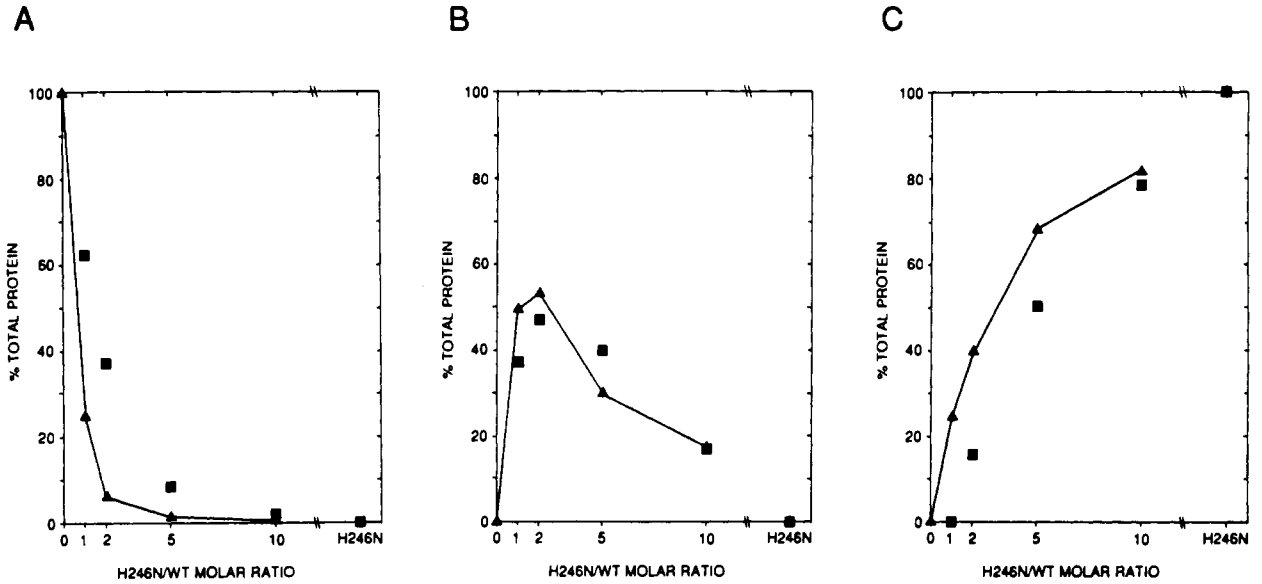

FIGURE 7: Quantitation of the relative amounts of tetramers in each enzyme mixture. Data were obtained from the densitometric scan of the Coomassie Blue stained gel shown in Figure 6. Symbols represent calculated values based on simple binomial distribution (▲) or observed values (■). Panel A, quantitation of wt tetramer species. Panel B, quantitation of hybrid tetramer species. Panel C, quantitation of H246N mutant tetramer species.

Table I: Analysis of Solutions Containing Refolded Mixtures of Wild-Type Succinyl-CoA Synthetase and Mutant H246N

H246N:wt molar ratio	total activity (%)			tetramer contribution to refolded protein activity[a]	
	calcd	−ATP	+ATP	wild type (%)	hybrid (%)
0	100	80.8	80.2	80.2	–
1	50	53.3	57.3	35.9	21.4
2	33.3	36.1	37.6	16.7	20.9
5	16.7	22.0	20.6	3.7	16.9
10	9.3	9.8	10.5	1.8	8.7
H246N alone	0	0	0	–	–

[a] Contributions of wild-type and hybrid tetramers were calculated from the data in Figure 7, with the assumption that wt and mutant α-subunits have the same probability of forming dimers with native β-subunits. Percentages are based on the starting wild-type enzyme activity.

Table II: Assay of Substrate Synergism Catalyzed by Mixtures of Refolded Wild-Type Succinyl-CoA Synthetase and Mutant H246N

H246N:wt molar ratio	rate of exchange[a] (μmol/min)	
	−ATP	+ATP
0	0.073 ± 0.008	1.43 ± 0.42
1	0.096 ± 0.032	1.50 ± 0.55
2	0.078 ± 0.019	1.38 ± 0.32
5	0.074 ± 0.019	1.20 ± 0.303
10	0.099 ± 0.046	1.24 ± 0.385
H246N alone	0.013 ± 0.006	0.036 ± 0.005

[a] Aliquots containing equivalent amounts of enzyme were taken from the mixtures described in Figure 6 for assay of succinate ↔ succinyl-CoA exchange. See Materials and Methods for details.

nearly identical and closely followed the calculated values of viable dimers in each enzyme mixture. These data are consistent with results obtained from hybrid enzymes containing chemically modified α-subunits (O'Connor-McCourt & Bridger, 1985). Data shown in Figures 6 and 7 were used to calculate the separate contribution of wt and hybrid tetramers to the total activity measured (Table I). At the molar ratio of 10 H246N to 1 wt, nearly all of the enzymatic activity was contributed by the hybrid tetramers present in this mixture.

Substrate Synergism. Enzyme mixtures refolded in the absence of ATP were used to measure the exchange rate of [^{14}C]succinate and succinyl-CoA. In these experiments, each enzyme mixture was assayed for exchange rate activity with or without added ATP. As previously demonstrated for wt enzyme (Bridger et al., 1968; Nishimura & Mitchell, 1984a), the presence of ATP greatly stimulated the rate of exchange of succinate and succinyl-CoA by enzyme mixtures. Exchange rates measured in the absence of ATP were all slightly above background (Table II). Reactions measured in the presence of ATP, however, resulted in similarly increased exchange rates for each of the enzyme mixtures tested, with the exception of the H246N refolded enzyme, which produced no appreciable exchange rate.

Discussion

We have replaced the active-site histidine residue, α-His^{246}, with an asparagine residue using site-directed mutagenesis techniques. The resulting mutant enzyme (designated H246N) was expressed, purified to homogeneity, and found to contain no enzymatic activity, as expected. The H246N mutant enzyme looked similar to wt enzyme, as judged by SDS–polyacrylamide gel electrophoresis (Figure 1A) and fluorescence spectroscopy (data not shown). However, slight migration differences were detected on native polyacrylamide gels (Figure 1B).

These differences were investigated further with isoelectric focusing techniques. Data obtained from these procedures showed that a homogeneous wt enzyme preparation, as determined by native and SDS gel electrophoresis, could be separated into three distinct bands (Figure 2A). Each of the three bands could be resolved into the component polypeptides of SCS (namely α- and β-subunits), as judged by two-dimensional SDS and urea–IEF gels (Figure 2, panels B and C). The distribution and additional α-subunit spots present in the wt sample detected on the two-dimensional urea–IEF gel (Figure 2C) gave our first indication of the separation of α-subunits based on their phosphorylation. In contrast, the H246N mutant enzyme migrated as a single band on native IEF gels (Figure 2A) that contained only one α- and β-subunit spot each, as judged by two-dimensional SDS and urea–IEF gels (Figure 2, panels D and E).

The three species present in wt enzyme preparations can be interconverted by using dephosphorylating or phosphorylating conditions (see Materials and Methods). It must be noted that wt enzyme, as isolated, varied in its phosphorylation

content from one enzyme preparation to another, as shown by comparison of lane 2 in Figure 2A to lane 1 in Figure 3. Incubation of wt enzyme with excess succinate and CoA to dephosphorylate the enzyme resulted in a majority of the protein being shifted to the slower migrating band (dephosphorylated species), as well as a small fraction of the protein migrating at the intermediate position (monophosphorylated species). Dephosphorylated wt enzyme incubated with [γ-^{32}P]ATP incorporated approximately 1.8 mol of [^{32}P]P_i/mol of SCS, indicating that both active-site histidines of this enzyme could be phosphorylated at the same time. Similar findings were first reported by Bowman and Nishimura (1975). These observations may account for the apparent incorporation of only 1 mol of [^{32}P]P_i/mol of SCS after incubation with [γ-^{32}P]ATP observed in previous reports (Leitzmann et al., 1970; Moffet et al., 1972; Ramaley et al., 1967) or of 1 mol or less of thiophosphoryl after incubation with [^{35}S]ATPγS (Wolodko et al., 1983; Nishimura & Mitchell, 1984b).

Conversion of diphosphorylated SCS to monophosphorylated and dephosphorylated enzyme was also measured by using excess CoA and increasing concentrations of succinate (Figure 4, panels A and B). Incubation of diphosphorylated SCS with CoA alone produced a decrease in band intensity (Figure 4B, lane 2), without a corresponding increase in nonphosphorylated enzyme. This is further evidence of the catalysis by SCS of CoA-stimulated enzyme-P $\leftrightarrow$ P_i exchange (Ramaley et al., 1967). The mechanism of this reaction is not understood. At the highest succinate concentration (25 μM), nearly all the diphosphorylated SCS has been converted to the mono- and dephosphorylated enzyme species. These data indicate that diphosphorylated SCS is converted to the dephosphorylated enzyme via a monophosphorylated enzyme intermediate.

As shown earlier in this study, purified SCS preparations could be separated into three species on the basis of their phosphorylation content by using native IEF gels. Enzyme mixtures containing increasing molar ratios of inactive H246N mutant enzyme to fully active wt enzyme were refolded in the presence of excess ATP (Figure 5). Samples of each mixture migrated as three distinct tetrameric species on native IEF gels with the following phosphoryl group content: wt tetramer, two phosphates; hybrid tetramer, one phosphate; H246N tetramer, zero phosphate (Figure 6).

Quantitation of the tetrameric species in each mixture demonstrated that the experimental data were similar to calculated tetramer values based on a simple binomial distribution. The slightly increased wt tetramer formation data (Figure 7A) were offset by the slightly decreased hybrid and H246N tetramer formation data (Figure 7, panels B and C). This balanced quantitation of tetrameric species may account for the close fit of the fractional activity data to data representing the calculated total amount of viable wt dimer units, whether they are present in wild-type or hybrid tetramers (Table I).

As shown in Table II, refolded mixtures were also used to test the stimulation of succinate $\leftrightarrow$ succinyl-CoA exchange by the addition of ATP. This phenomenon, referred to as substrate synergism, had been previously observed for wt enzyme (Bridger et al., 1968; Nishimura & Mitchell, 1984a). Data from these experiments showed that each enzyme mixture tested produced exchange rates similar to that of refolded wt enzyme alone, even in the case when most of the total enzymatic activity was contributed by hybrid tetramers (10:1 enzyme molar ratio; see Table I). From these data, we have concluded that substrate synergistic effects occur at the "same site" in which all ligands are bound at the active site. This conclusion is supported by similar results obtained with the dimeric pig heart SCS (Nishimura & Mitchell, 1985), which contains only one active site.

Recently, Buck and Guest (1989) have reported the replacement of the active-site histidine residue, α-His^{246}, with an aspartic acid residue using site-directed mutagenesis techniques to ultimately aid in the separation of hybrid enzymes based on charge differences. In contrast to data reported in this study, these workers were unable to show any significant expression of the resulting mutant protein in cellular extracts, indicating that the less conservative replacement of the histidine side chain with the negatively charged aspartic acid side chain was detrimental to the enzyme.

In summary, our data show that replacement of an active-site histidine residue of *E. coli* succinyl-CoA synthetase with an asparagine residue resulted in a completely inactive mutant enzyme, as expected. Wild-type SCS is partially phosphorylated as isolated and can be separated into diphosphorylated, monophosphorylated, and unphosphorylated species on native IEF polyacrylamide gels. These distinct forms of SCS can be interconverted upon incubation with substrates. Hybrid tetramers ($\alpha\beta\alpha^{H246N}\beta$) exhibit enzymatic activity and substrate synergism, indicating that the tetrameric form of *E. coli* SCS is comprised of two independently active dimer molecules associated together to form a "dimer of dimers" that displays substrate synergism within each dimer and not necessarily between dimers. Incubation of SCS with [γ-^{32}P]ATP after dephosphorylation led to the incorporation of close to 2 mol of ^{32}P/mol of SCS. While kinetic differences in the incorporation of the two phosphoryl groups cannot be ruled out, the conclusion that SCS is a half of sites reactive protein based on phosphorylation measurements of the native enzyme is called into question. Taken together, the observations described in this study do not support the notion that catalysis of the overall reaction by *E. coli* SCS proceeds by a mechanism in which the two active sites function in an alternating manner.

Further studies of the mutant enzyme H246N are in progress. The stability of this unphosphorylated enzyme is interesting, since wild-type SCS is marginally stable in the dephosphorylated state (Moffet et al., 1972). A complete study of binding of ligands to this enzyme will be performed and should reveal whether the inability of the enzyme to phosphorylate itself affects other processes that one might expect to be normal. Curiously, while CoA binds to H246N, this enzyme does not catalyze CoA $\leftrightarrow$ succinyl-CoA exchange, as predicted from eq 2c (Nishimura et al., unpublished data).

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Registry No. SCS, 9080-33-5; L-His, 71-00-1; ATP, 56-65-5; succinyl-CoA, 604-98-8; succinic acid, 110-15-6.

REFERENCES

Atkinson, T., & Smith, M. (1984) in *Oligonucleotide Synthesis: A Practical Approach* (Gait, M. J., Ed.) pp 35–81, IRL Press, Arlington, VA.

Bild, G. S., Janson, C. A., & Boyer, P. D. (1980) *J. Biol. Chem. 255*, 8109–8115.

Blackshear, P. J. (1984) *Methods Enzymol. 104*, 237–255.

Bowman, C. M., & Nishimura, J. S. (1975) *J. Biol. Chem. 250*, 5609–5613.

Bridger, W. A. (1971) *Biochem. Biophys. Res. Commun. 42*, 948–954.
Bridger, W. A. (1974) in *The Enzymes* (Boyer, P. D., Ed.) Vol. 10, pp 581–606, Academic press, Orlando, FL.
Bridger, W. A., Millen, W. A., & Boyer, P. D. (1968) *Biochemistry 7*, 3608–3616.
Buck, D., & Guest, J. R. (1989) *Biochem. J. 260*, 737–747.
Buck, D., Spencer, M. E., & Guest, J. R. (1985) *Biochemistry 24*, 6245–6252.
Collier, G. E., & Nishimura, J. S. (1978) *J. Biol. Chem. 253*, 4938–4943.
Grinnell, F. L., & Nishimura, J. S. (1969) *Biochemistry 8*, 562, 568.
Kaufman, S. (1955) *J. Biol. Chem. 216*, 153–164.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Leitzmann, C., Wu, J.-Y., & Boyer, P. D. (1970) *Biochemistry 9*, 2338–2346.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Maniatis, T., Fritsch, E. J., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Mann, C. J., Hardies, S. C., & Nishimura, J. S. (1989) *J. Biol. Chem. 264*, 1457–1460.
Moffet, F. J., Wang, T.-T., & Bridger, W. A. (1972) *J. Biol. Chem. 247*, 8139–8144.
Nishimura, J. S. (1986) *Adv. Enzymol. Relat. Areas Mol. Biol. 58*, 142–172.
Nishimura, J. S., & Grinnell, F. (1972) *Adv. Enzymol. Relat. Areas Mol. Biol. 36*, 183–202.
Nishimura, J. S., & Mitchell, T. (1984a) *J. Biol. Chem. 259*, 2144–2148.
Nishimura, J. S., & Mitchell, T. (1984b) *J. Biol. Chem. 259*, 9642–9645.
Nishimura, J. S., & Mitchell, T. (1985) *J. Biol. Chem. 260*, 2077–2079.
O'Connor-McCourt, M. D., & Bridger, W. A. (1985) *Can. J. Biochem. Cell Biol. 63*, 57–63.
Pearson, P. H., & Bridger, W. A. (1975) *J. Biol. Chem. 250*, 8524–8529.
Ramaley, R. F., Bridger, W. A., Moyer, R. W., & Boyer, P. D. (1967) *J. Biol. Chem. 242*, 4287–4298.
Robertson, E. F., Dannelly, H. K., Malloy, P. J., & Reeves, H. C. (1987) *Anal. Biochem. 167*, 290–294.
Vogel, H. J., & Bridger, W. A. (1982) *J. Biol. Chem. 257*, 4834–4842.
Wolodko, W. T., Brownie, E. R., O'Connor, M. D., & Bridger, W. A. (1983) *J. Biol. Chem. 258*, 14116–14119.

5′-Nucleotidase I from Rabbit Heart†

Yukiko Yamazaki, Vu L. Truong, and John M. Lowenstein*
Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254
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ABSTRACT: 5′-Nucleotidase I (N-I) from rabbit heart was purified to homogeneity. After ammonium sulfate precipitation, the purification involved chromatography on phosphocellulose, DEAE-Sepharose, AMP–agarose, and ADP–agarose. The pure enzyme has a specific activity of 318 μmol (mg of protein)$^{-1}$ min^{-1}. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate yields a subunit molecular weight of 40 000. N-I is activated by ADP but not by ATP, in contrast to the 5′-nucleotidase (N-II) purified by Itoh et al. (1986), which is activated by ATP and, less well, by ADP. N-I displays sigmoidal saturation kinetics in the absence of ADP and hyperbolic kinetics in the presence of ADP. Partially purified N-I was previously shown to prefer AMP over IMP as substrate (Truong et al., 1988); this has been confirmed for pure N-I. Comparison of AMP and ADP concentrations reported to occur in heart with the kinetic behavior of N-I implicates N-I as the enzyme responsible for producing adenosine under conditions leading to a rise in ADP and AMP, such as hypoxia or increased workload. N-I is not activated by the ADP analogue adenosine 5′-methylenediphosphonate (AOPCP) and is only weakly inhibited by relatively high concentrations of AOPCP, in contrast to 5′-nucleotidase from plasma membrane, which is powerfully inhibited by this analogue. N-I shows an absolute dependence on Mg^{2+} ions. Mn^{2+} and Co^{2+} ions can replace Mg^{2+} ions as activator; Ni^{2+} and Fe^{2+} are much less effective, while Ca^{2+}, Ba^{2+}, Zn^{2+}, and Cu^{2+} fail to activate the enzyme.

Adenosine plays an important role in regulating coronary blood flow (Berne, 1980). It is released from heart cells during exercise, hypoxia, and ischemia and acts as a vasodilator. Adenosine is formed by the 5′-nucleotidase reaction and is destroyed principally by the adenosine deaminase reaction. In rat heart, the most active 5′-nucleotidase is the plasma membrane enzyme. The regulatory properties of this enzyme, such as its powerful inhibition by ADP (Naito & Lowenstein, 1981, 1985), as well as its outward-facing orientation, make it unlikely that it is the enzyme responsible for adenosine production under conditions when heart muscle increases its blood supply by using adenosine as a signal for coronary vasodilation. Moreover, studies of adenosine formation by heart cells in culture, in the presence and absence of inhibitors of adenosine transport, indicate that adenosine is formed intracellularly under these conditions (Meghji et al., 1985, 1988a,b; Altschuld et al., 1987). A cytosolic 5′-nucleotidase was isolated by Itoh and co-workers and thought to be responsible for generating intracellular adenosine (Itoh & Oka, 1985; Itoh et al., 1986). However, this enzyme has a much higher affinity for IMP than for AMP and is activated strongly by ATP and less strongly by ADP. In the heart, $[ADP]_{free}$ rises substantially in response to increased workload, hypoxia, and ischemia, while [ATP] drops. The enzyme responsible for producing adenosine under

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