7140 *Biochemistry* **2009,** *48,* 7140–7149
DOI: 10.1021/bi900725c

BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Succinyl-CoA Synthetase Is a Phosphate Target for the Activation of Mitochondrial Metabolism[†]

Darci Phillips,[‡] Angel M. Aponte,[§] Stephanie A. French,[‡] David J. Chess,[‡] and Robert S. Balaban*,[‡]

[‡]*Laboratory of Cardiac Energetics and* [§]*Proteomics Core Facility, National Heart, Lung, and Blood Institute, National Institutes of Health, Department of Health and Human Services, Bethesda, Maryland 20892*

Received April 27, 2009; Revised Manuscript Received June 14, 2009

ABSTRACT: Succinyl-CoA synthetase (SCS) is the only mitochondrial enzyme capable of ATP production via substrate level phosphorylation in the absence of oxygen, but it also plays a key role in the citric acid cycle, ketone metabolism, and heme synthesis. Inorganic phosphate (P_i) is a signaling molecule capable of activating oxidative phosphorylation at several sites, including NADH generation and as a substrate for ATP formation. In this study, it was shown that P_i binds the porcine heart SCS α-subunit (SCSα) in a noncovalent manner and enhances its enzymatic activity, thereby providing a new target for P_i activation in mitochondria. Coupling ^{32}P labeling of intact mitochondria with SDS gel electrophoresis revealed that ^{32}P labeling of SCSα was enhanced in substrate-depleted mitochondria. Using mitochondrial extracts and purified bacterial SCS (BSCS), we showed that this enhanced ^{32}P labeling resulted from a simple binding of ^{32}P, not covalent protein phosphorylation. The ability of SCSα to retain its ^{32}P throughout the SDS denaturing gel process was unique over the entire mitochondrial proteome. In vitro studies also revealed a P_i-induced activation of SCS activity by more than 2-fold when mitochondrial extracts and purified BSCS were incubated with millimolar concentrations of P_i. Since the level of ^{32}P binding to SCSα was increased in substrate-depleted mitochondria, where the matrix P_i concentration is increased, we conclude that SCS activation by P_i binding represents another mitochondrial target for the P_i-induced activation of oxidative phosphorylation and anaerobic ATP production in energy-limited mitochondria.

Protein phosphorylation is an important regulatory mechanism in the cytosol and mitochondrial matrix. ^{32}P labeling of intact mitochondria, coupled to sodium dodecyl sulfate (SDS) gel electrophoresis, is a useful technique for identifying phosphoproteins that actively undergo phosphate exchange in the matrix (*1*−*4*). Previous studies from our lab revealed the dynamic nature of the mitochondrial phosphoproteome by exposing intact porcine heart mitochondria to exogenous ^{32}P, which was transported into the matrix and converted to [γ-^{32}P]ATP for protein phosphorylation (*1*, *2*, *4*). Naturally, mitochondria must be energized to generate [γ-^{32}P]ATP via oxidative phosphorylation. The current work initially sought to screen for the energy-signaling systems within mitochondria by evaluating whether specific changes in ^{32}P incorporation occurred in substrate-depleted mitochondria, where the matrix ATP concentration, membrane polarization, and NADH concentration are low and matrix inorganic phosphate and ADP concentrations are high. As shown here, the most pronounced result of these studies was the enhanced ^{32}P labeling of the α-subunit of succinyl-CoA synthetase (SCSα)[1] in substrate-depleted mitochondria. The remainder of this work aimed to characterize the mechanism of strengthened association of ^{32}P with SCSα and evaluate phosphate's effect on succinyl-CoA synthetase (SCS) activity.

SCS is a Krebs cycle enzyme that catalyzes substrate-level phosphorylation in the forward direction (*5*) and replenishes succinyl-CoA for ketone body catabolism (*6*) and porphyrin biosynthesis (*7*) in the reverse direction. In mammals, two isoforms of SCS have been identified: one specific for ADP/ATP and another specific for GDP/GTP. SCS consists of a highly conserved α-subunit and a *β*-subunit that determines nucleotide specificity between ATP and GTP. The α*β* complex is required for the SCS reaction to occur in either direction. In highly oxidative tissues, such as heart and skeletal muscle, the ATP-specific *β*-subunit transcript is more strongly expressed, whereas in tissues serving a biosynthetic role, such as liver, the GTP-specific transcript is preferential (*8*, *9*). In some species (i.e., *Escherichia coli*), SCS is not

[†]These studies were funded by the National Institutes of Health Division of Intramural Research.

*To whom correspondence should be addressed: Laboratory of Cardiac Energetics, National Heart, Lung, and Blood Institute, National Institutes of Health, 10 Center Dr., Room B1D416, Bethesda, MD 20892-1061. Telephone: (301) 496-3658. Fax: (301) 402-2389. E-mail: rsb@nih.gov.

[1]Abbreviations: SCS, succinyl-CoA synthetase; SCSα, succinyl-CoA synthetase α-subunit; BSCS, bacterial succinyl-CoA synthetase; BSCSα, bacterial succinyl-CoA synthetase α-subunit; P_i, inorganic phosphate; RCR, respiratory control ratio; G/M, potassium glutamate and potassium malate; TCA, trichloroacetic acid; P_iC, mitochondrial phosphate carrier protein; IEV, isoelectric variant; BN-PAGE, blue-native polyacrylamide gel electrophoresis.

 Published on Web 06/15/2009

specific, using either ATP or GTP (*8*). SCSα has a well-documented histidine phosphorylation site (*10*−*14*) but has also been shown to bind phosphate in its dephosphorylated form as a means of stabilizing the complex (*8*). Furthermore, SCS is the only enzyme capable of generating ATP in the absence of a proton motive force in the inner membrane, potentially playing a role in maintaining matrix ATP levels under energy-limited conditions, such as transient hypoxia (*15*−*17*).

Inorganic phosphate (P_i), a putative cytosolic signaling molecule, plays a multifaceted role in the regulation of mitochondrial metabolism. P_i can alter the free concentration of Mg^{2+} and Ca^{2+} ions (*18*−*20*), increase mitochondrial volume (*21*−*23*), influence the mitochondrial transition pore (*24*, *25*), and directly modify the activity of several Krebs cycle dehydrogenases (*26*−*29*). In a series of studies on intact mitochondria, Bose et al. (*30*) showed that extramitochondrial P_i modulates oxidative phosphorylation at several levels, including via substrate oxidation, via membrane potential generation, and through its well-known effect as a substrate for ATP synthesis in Complex V. However, the specific sites of P_i-induced activation for substrate oxidation in mitochondria have not been completely identified (*30*).

The purpose of this study was to evaluate the mechanism of associationof P_i with SCS under energy-limited conditions and to determine if SCS is another P_i target for activating mitochondrial energetics. To accomplish this task, ^{32}P labeling of energized and substrate-depleted intact porcine heart mitochondria, protein extracts, and purified bacterial SCS (BSCS) was studied with a variety of proteomics approaches. After establishing that simple P_i binding was responsible for the enhanced ^{32}P labeling of SCSα in energy-limited mitochondria, we determined the regulatory effect of P_i on SCS activity using in-solution and in-gel assays in mitochondrial homogenates and purified BSCS.

MATERIALS AND METHODS

Materials. Salts and inorganic phosphate (P_i) were purchased from Sigma (St. Louis, MO). ^{32}P (10 mCi/mL) and [γ-^{32}P]ATP (10 mCi/mL) were purchased from PerkinElmer (Boston, MA). Unless otherwise stated, two-dimensional (2D) gel electrophoresis reagents, equipment, and software were purchased from GE Healthcare (Piscataway, NJ).

Isolation of Porcine Heart Mitochondria. All procedures were performed in accordance with the guidelines described in the Animal Care and Welfare Act (7 USC 2142 § 13) and approved by the NHLBI Animal Care and Use Committee. Porcine heart mitochondria were isolated as previously described (*1*). Unless otherwise noted, mitochondria were briefly exposed to 1 mM P_i during the isolation process to prevent matrix P_i depletion. Mitochondrial preparations were tested for viability by measuring the respiratory control ratio (RCR); to be accepted, the RCR had to reach at least 8 (*31*). The mitochondrial protein concentration was determined using the USB Quant Kit (USB Corp., Cleveland, OH).

^{32}P Labeling of Intact Mitochondria. The standard experimental buffer for our studies was buffer A, composed of 125 mM KCl, 15 mM NaCl, 20 mM HEPES, 1 mM EGTA, 1 mM EDTA, and 5 mM $MgCl_2$ (pH 7.1). The pH was titrated separately at room temperature and 37 °C. P_i was added as the potassium salt, pretitrated to pH 7.1. To screen for protein phosphorylation, we added ^{32}P to intact porcine heart mitochondria, as previously described (*1*). Briefly, mitochondria (1 mg/mL) were incubated with ^{32}P (250 μCi/mg of protein) in oxygenated buffer A for 20 min at 37 °C. Energized mitochondria were incubated with 5 mM potassium glutamate and 5 mM potassium malate (G/M); substrate-depleted mitochondria were incubated without carbon substrates. Unless otherwise stated, incorporation of ^{32}P was quenched by adding an equal volume of 10% trichloroacetic acid (TCA). For non-acid experiments, 100% methanol replaced TCA.

^{32}P Labeling of Mitochondrial Homogenates and Purified SCS. Mitochondria were solubilized to a concentration of 10 mg/mL in buffer A with 1% dodecyl maltoside detergent and placed on ice for 30 min. Solubilized mitochondria and purified bacterial SCS (BSCS) from *E. coli* (Megazyme International, Wicklow, Ireland) were incubated with ^{32}P at room temperature for 30 min, under a variety of conditions. Purified BSCS was used to confirm P_i binding in these studies because it was commercially available at high purity. It is important to note that the BSCS α-subunit (BSCSα) displays a large degree of amino acid sequence conservation with mammalian forms (*8*, *32*). Prior to the ^{32}P incubation, purified BSCS was diluted with an equal volume of buffer A and added to 5 μg of bovine serum albumin (BSA) to minimize protein loss during cleanup.

Purification and ^{32}P Labeling of the Mitochondrial Phosphate Carrier. The mitochondrial phosphate carrier (P_iC) was purified from porcine heart mitochondria, as previously described (*33*). In addition to the P_iC, two other bands (identified as the α- and β-subunits of Complex V) were components of the preparation. The P_iC was incubated with ^{32}P, as described above for purified SCS.

Two-Dimensional SDS Gel Electrophoresis. Proteins were cleaned using a standard TCA/acetone protocol and resuspended in lysis buffer [15 mM Tris-HCl, 7 M urea, 2 M thiourea, and 4% CHAPS (w/v)] to a final concentration of approximately 10 mg/mL. Two-hundred micrograms of sample was mixed with rehydration solution [7 M urea, 2 M thiourea, 4% CHAPS (w/v), 13 mM DTT, 1% (pH 3−11NL or pH 7−11NL) Pharmalyte (v/v), and 2 μL of Destreak reagent] to a final volume of 210 μL and placed on ice for 5 min before being loaded onto 11 cm Immobiline DryStrip gels (pH 3−11NL or pH 8−11, Sigma-Aldrich, St. Louis, MO). For pH 3−11NL Immobiline DryStrip gels, isoelectric focusing was achieved by active rehydration for 12 h at 30 V followed by stepwise application of 500 V (1 h), 1000 V (1 h), a gradient to 6000 V (2 h), and a final step at 7550 V (1.2 h) for a total of ∼15000 V h (Ettan IPG Phor2). For pH 8−11 Immobiline DryStrip gels, isoelectric focusing was achieved by active rehydration for 12 h at 30 V followed by stepwise application of 500 V (1 h), 1000 V (1 h), a gradient to 6000 V (2 h), and a final step at 65000 V (11 h) for a total of ∼78000 V h.

Immobiline DryStrip gels were then equilibrated in 5 mL of SDS equilibration solution [50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, and 2% SDS] for 10 min, first containing 100 mg of DTT and then 250 mg of iodoacetemide. Gel strips were applied to 8−16% Tris-HCl gels (Bio-Rad Laboratories, Hercules, CA), and electrophoresis was performed in a Criterion Cell (Bio-Rad), with SDS electrophoresis buffer [25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 0.2% SDS] for ∼210 V h. Gels were stained for 2 h with Coomassie Blue Solution [50% methanol, 3% phosphoric acid, and 0.05% (w/v) Coomassie Blue G-250 (Bio-Rad)], followed by destaining for 2 h with 30% methanol and 3% phosphoric acid. For non-acid experiments, acid was omitted from the Coomassie Blue and destain solutions. Gels were dried, exposed to a phosphor screen for 24 h, and scanned

on a Typhoon 9410 variable mode imager at a resolution of 100 μm, as previously described (*1*).

One-Dimensional SDS Gel Electrophoresis. Various amounts of solubilized porcine heart mitochondria, purified BSCS, and purified mitochondrial P_iC were labeled with ^{32}P at room temperature for 30 min. To ensure that the binding of ^{32}P to BSCS was not an artifact of recombinant proteins, an equivalent amount of two additional purified recombinant proteins from *E. coli* [Complex I 30 kDa subunit and Peroxiredoxin 3 (GenWay Biotech, San Diego, CA)] was incubated with ^{32}P at room temperature for 30 min. ^{32}P labeling was quenched with tricine sample buffer (Bio-Rad Laboratories) and run on a 16.5% tricine gel (Bio-Rad Laboratories) with tricine-SDS electrophoresis buffer [100 mM Tris-HCl (pH 8.3), 100 mM tricine, and 0.2% SDS] for ~190 V h. The gel was stained, dried, exposed, and imaged as described above.

Blue-Native PAGE. Solubilized porcine heart mitochondria and purified BSCS were incubated with ^{32}P as described above. The reaction was quenched by adding an equal volume of 2× Native PAGE buffer (Invitrogen, Carlsbad, CA) with 1% dodecyl maltoside. Samples were then processed for blue native (BN)-PAGE as previously described (*34*), with 100 μg of solubilized mitochondria and 10 μg of BSCS loaded per well. After electrophoresis, gels were cut into individual BN-PAGE lanes and dried immediately or run in the second dimension. For 2D BN-PAGE, lanes were incubated in 5 mL of SDS equilibration solution with 100 mg of DTT for 10 min, applied to 10 to 15% SDS−PAGE gels (Nextgen Sciences, Ann Arbor, MI), and sealed with 0.5% agarose containing bromophenol blue. Electrophoresis was performed in an Ettan DALT-12 tank in 20 °C SDS electrophoresis buffer for ~2150 V h. Gels were then stained with Coomassie Blue, dried, exposed, and imaged as described above.

Mass Spectrometry Identifications. Protein identifications were obtained in paired experiments from nonradioactive gels. Protein spots were manually picked using a 1.5 mm spot picker plus system (The gel Company, San Francisco, CA). Each protein spot was trypsin digested as previously described (*35*) and prepared for liquid chromatography−tandem mass spectroscopy (LC−MS/MS) analysis. In descending order, peptide digests 7 to 1 were analyzed using an LTQ-Orbitrap XL mass spectrometer (ThermoFisher, San Jose, CA). In each data collection cycle, one full high-resolution FTMS scan (m/z 300−2000) was acquired in the Orbitrap, and then the six most abundant ions per cycle were fragmented and analyzed in the linear trap. Database searching was performed using the Mascot search engine, version 2.2 (Matrix Science, Boston, MA). All MS/MS data sets were searched against the Swiss Prot mammal database, version 14.9, with 64199 sequences (Swiss Institute of Bioinformatics). Protein modifications were selected as carbamidomethyl (C) (fixed) and oxidation (M) (variable). Up to one missed cleavage was allowed.

Measurement of Succinyl-CoA Synthetase Activity. SCS activity was assayed in the direction of succinyl-CoA formation by measuring the rate of ATP hydrolysis in solubilized porcine heart mitochondria and purified BSCS. The SCS reaction was conducted in the reverse direction to prevent the substrate effects of P_i in the forward (ATP synthetic) direction. Porcine heart mitochondria were solubilized as described above, and 1 mg was incubated with P_i (from 0 to 20 mM) for 30 min at room temperature. Using a procedure modified from a previously described spectrophotometric method (*36*), samples were added to 1 mL of assay buffer, containing 50 mM succinate, 10 mM $MgCl_2$, 0.3 mM CoA, 100 mM Tris (pH 7.5), and 15 μg of oligomycin B per milliliter. After incubation for 2 min at 30 °C, the assay was started with the addition of 0.5 mM ATP. Reactions were quenched via addition of 150 μg of sample to an equal volume of cold 6% perchloric acid at 0, 1, 2.5, 5, 7.5, and 10 min. After 10 min, samples were neutralized with 3 M K_2CO_3, in the presence of pH indicator solution. The ATP content was then measured using a luciferin-luciferase kit (Invitrogen) with a standard curve of ATP. To ensure that ATP consumption was succinate-dependent, a sample without succinate and CoA was assayed; ATP consumption in this background sample was negligible. The activity of purified BSCS was assessed using the same method, with 100 μg of starting material and measuring ATP content in 15 μg of sample at the various time points.

SCS activity was also assayed using a newly developed BN-PAGE method to quantify ATP and GTP hydrolysis. One hundred micrograms of purified SCS was diluted with 10 μL of buffer A and incubated with P_i concentrations ranging from 0 to 10 mM for 30 min at room temperature. The reaction was quenched by adding 20 μL of 2× Native PAGE buffer (Invitrogen) with 1% dodecyl maltoside. Samples were processed for BN-PAGE as described above, with 10 μg of protein loaded per well. Using BN-PAGE is advantageous because it allows the SCS complex to remain in its intact, active form (*37*). After electrophoresis, gels were incubated in buffer B [35 mM Tris-HCl (pH 7.5), 14 mM $MgSO_4$, and 270 mM glycine (pH 7.8)] for 30 min at room temperature. Gels were then transferred to buffer C [35 mM Tris-HCl (pH 7.5), 14 mM $MgSO_4$, 270 mM glycine, 0.2% (w/v) $Pb(NO_3)_2$, 0.5 mM CoA, and 20 mM succinate (pH 7.8)] with either 8 mM ATP or 8 mM GTP, for 1.5 h at room temperature. Gels were fixed for 5 min in a solution containing 30% methanol and 3% phosphoric acid and then imaged against a Coomassie blue-colored background with infrared lights and a red filter (Wratten #25, Kodak, Rochester, NY). Images were converted to grayscale, and bands were quantified using ImageQuant TL (GE Healthcare).

Statistical Analysis. The data were analyzed using a Student's t test. A p value of < 0.01 was considered statistically significant.

RESULTS

Two basic objectives were evaluated in this study: to characterize the mechanism of association of ^{32}P with SCSα using SDS gel electrophoresis and to evaluate the functional significance of bindingof P_i to SCS.

Effects of the Mitochondrial Energetic State on ^{32}P Labeling. A comparison of porcine heart mitochondrial proteins labeled with ^{32}P under energized and substrate-depleted conditions was evaluated using 2D gel electrophoresis. Substrate-depleted mitochondria represent a low-energy state, meaning that these mitochondria have a membrane potential but are unable to support sustained ATP production. Specifically, these substrate-depleted mitochondria have a membrane potential of approximately −80 mV and an NADH/NAD ratio of < 0.10, compared to values of approximately −175 mV and 0.65, respectively, in mitochondria supplied with 5 mM glutamate and 5 mM malate (G/M). A comparison of ^{32}P-labeled mitochondria under different energetic states is presented in Figure 1. Mitochondria energized with G/M showed ^{32}P incorporation for hundreds of proteins (Figure 1A). However, substrate-depleted mitochondria revealed a decreased level of ^{32}P labeling for a

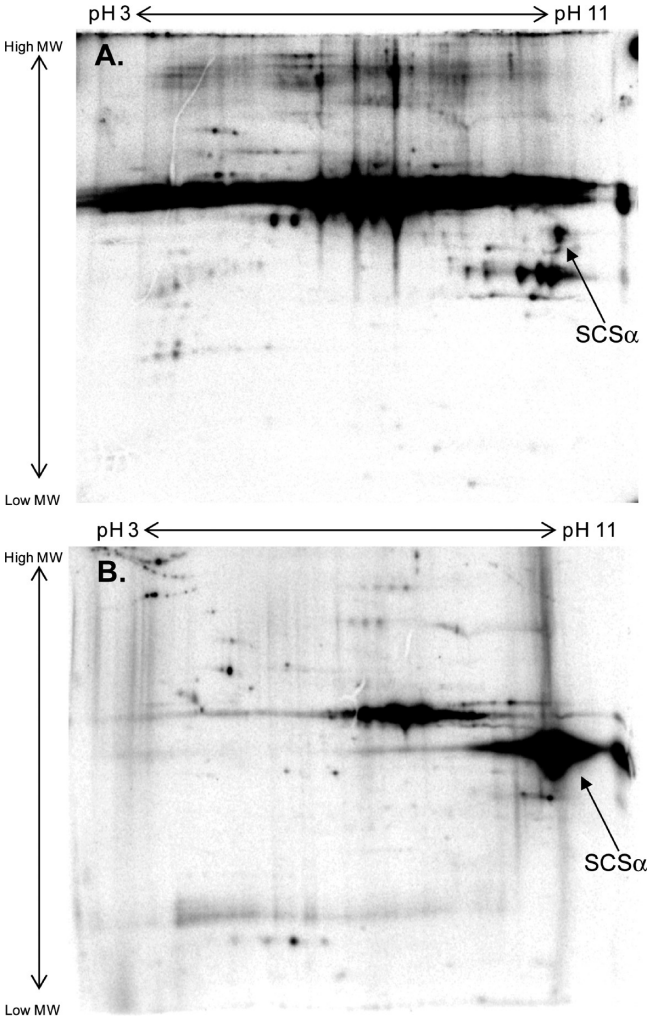

FIGURE 1: Energy-sensitive ^{32}P association for the succinyl-CoA synthetase α-subunit. Intact porcine heart mitochondria were labeled with ^{32}P under energized conditions with G/M (A) or substrate-depleted conditions without carbon substrates (B). Proteins were first separated by isoelectric focusing point, over a pH range of 3−11NL, and then by molecular mass from ~150 to 10 kDa. The arrow in each panel highlights the ^{32}P labeling of the succinyl-CoA synthetase α-subunit following 2D SDS gel electrophoresis, where SCSα represents porcine heart mitochondrial the succinyl-CoA synthetase α-subunit.

majority of proteins, with the exception of SCSα which showed an increased level of ^{32}P incorporation (Figure 1B). Mass spectrometry was used to identify this protein as SCSα in a paired, nonradioactive study.

To further evaluate the mechanism of association of ^{32}P with SCSα in substrate-depleted mitochondria, several labeling experiments were conducted with solubilized porcine heart mitochondrial protein. To screen for kinase-mediated protein phosphorylation, including autophosphorylation, 400 μg of solubilized mitochondria was incubated with 40 μCi of [γ-^{32}P]ATP for 30 min at room temperature. As observed in the intact ^{32}P labeling studies described above, intense ^{32}P incorporation was found in SCSα (Figure 2A). Since commercial preparations of [γ-^{32}P]ATP contain a degree of free ^{32}P and [γ-^{32}P]ATP is hydrolyzed to ADP and $^{32}P_i$ in mitochondrial homogenates, it is difficult to determine whether the radiolabeling is dependent on ATP or P_i. To evaluate the source of this binding, we also incubated solubilized mitochondria with a mixture of [γ-^{32}P]ATP and 5 mM P_i in a parallel experiment (Figure 2B). In the presence

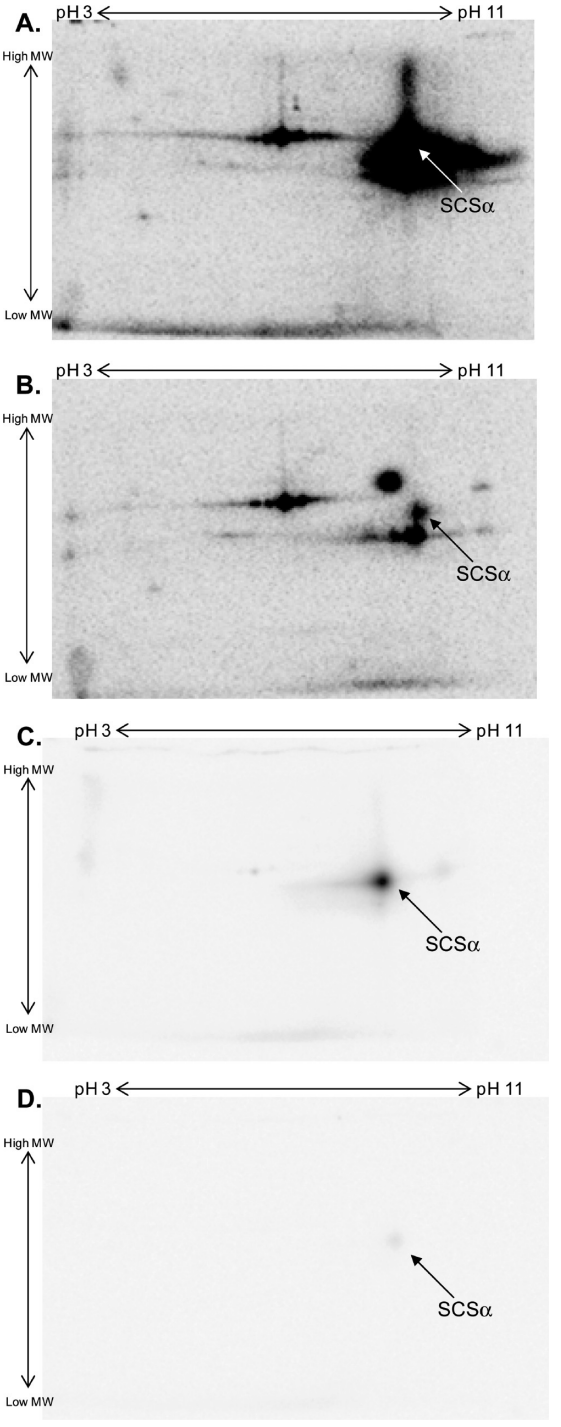

FIGURE 2: Enhanced ^{32}P labeling by succinyl-CoA synthetase in solubilized mitochondria. Solubilized porcine heart mitochondria incubated with [γ-^{32}P]ATP (A) or a mixture of [γ-^{32}P]ATP and 5 mM P_i (B). The arrows in panels A and B refer to the succinyl-CoA synthetase α-subunit. Solubilized porcine heart mitochondria incubated with ^{32}P (C) or a mixture of ^{32}P and 5 mM P_i (D). Proteins were first separated by isoelectric focusing point, over a pH range of 3−11NL, and then by molecular mass from ~150 to 10 kDa. In all panels, an arrow is used to indicate the binding of ^{32}P to succinyl-CoA synthetase following 2D SDS gel electrophoresis, where SCSα represents the porcine heart mitochondrial succinyl-CoA synthetase α-subunit.

of 5 mM P_i, the level of ^{32}P labeling of SCS was decreased, suggesting that a majority of its ^{32}P incorporation resulted from a simple binding of P_i. Covalent bond formation requires energy. In the absence of an additional energy source, the only way that

P_i can participate in this reaction is via a noncovalent binding mechanism. To confirm a noncovalent binding of P_i to SCSα, 400 μg of solubilized mitochondrial protein was incubated with 40 μCi of ^{32}P for 30 min at room temperature. As shown in Figure 2C, SCSα was the only mitochondrial protein that bound ^{32}P in a manner that persisted throughout exposure to the denaturing properties of SDS. Furthermore, incubating solubilized mitochondria with a mixture of 40 μCi of ^{32}P and 5 mM P_i (Figure 2D) or incubating mitochondria with 40 μCi of ^{32}P for 15 min followed by 5 mM P_i for an additional 15 min (results not shown) displaced most of SCSα's ^{32}P labeling, consistent with a simple P_i binding mechanism. It is important to point out that we were unable to exchange out the ^{32}P bound to SCSα by soaking the SDS-containing gel in a 5 mM P_i solution for 3 h. This result revealed that the binding of ^{32}P to SCS was labile in the native protein, but extremely tight following denaturation with SDS. This "trapping" of phosphate by the SDS treatment explains why the ^{32}P label can persist for hours in solutions containing essentially zero P_i during the SDS gel process.

Confirmation of the P_i-Binding Protein as Succinyl-CoA Synthetase. With an isoelectric focusing point (pI) of ~9.5 and a molecular mass of ~35 kDa, SCSα is located in a very complex region of the 2D gel. To confirm the identity of this ^{32}P-binding protein as SCSα, mass spectrometry was performed on a paired, nonradioactive gel that separated proteins across a narrow pH range (8−11) (Figure 1 of the Supporting Information). Additionally, a stoichiometric amount of purified BSCS (15 μg) was incubated with 40 μCi of ^{32}P for 30 min at room temperature. As observed for solubilized porcine heart mitochondria (Figure 3A), purified BSCS also revealed intense ^{32}P incorporation for the conserved α-subunit (Figure 3B). Notably, purified BSCSα had a slightly lower molecular mass and a more acidic pI than porcine SCSα. These differences were utilized to conduct a mix experiment, where 400 μg of solubilized porcine heart mitochondria and 15 μg of purified BSCS were incubated together with 40 μCi of ^{32}P for 30 min at room temperature. Even in this complex mixture (Figure 3C), both forms of SCSα were labeled with ^{32}P, underlining the identification of the porcine protein as SCSα.

Since the mitochondrial P_i carrier (P_iC) protein binds P_i as a component of its mechanism and has properties very similar to those of SCSα (molecular mass of ~35 kDa and pI of ~9.5) (*38*−*40*), there was some concern that the ^{32}P binding observed in this study was the result of underlying P_iC, and not SCSα. To determine whether the P_iC bound ^{32}P in a manner that survived SDS gel electrophoresis, up to 25 μg of purified P_iC was incubated with 40 μCi of ^{32}P for 30 min at room temperature. As shown in Figure 4, ^{32}P binding to P_iC did not survive 1D or 2D SDS gel electrophoresis. Thus, these results confirmed that SCSα, and not the mitochondrial P_iC, was responsible for the enhanced ^{32}P binding observed in substrate-depleted mitochondria. Furthermore, to ensure that the association of ^{32}P with BSCS was not an artifact of recombinant proteins, purified Complex I 30 kDa subunit and peroxiredoxin-3 were incubated with ^{32}P under the same conditions; no ^{32}P binding was observed (results not shown).

Effect of SDS and pH on the Extent of ^{32}P Binding to Succinyl-CoA Synthetase. To examine the effect of denaturing agents, such as SDS and acid, on the level of ^{32}P binding to SCSα, two paired experiments were conducted. First, ^{32}P-labeled solubilized porcine heart mitochondria and purified BSCS were run using BN-PAGE, thereby maintaining the SCS protein complex in its intact form. As shown in Figure 5A, the extent of

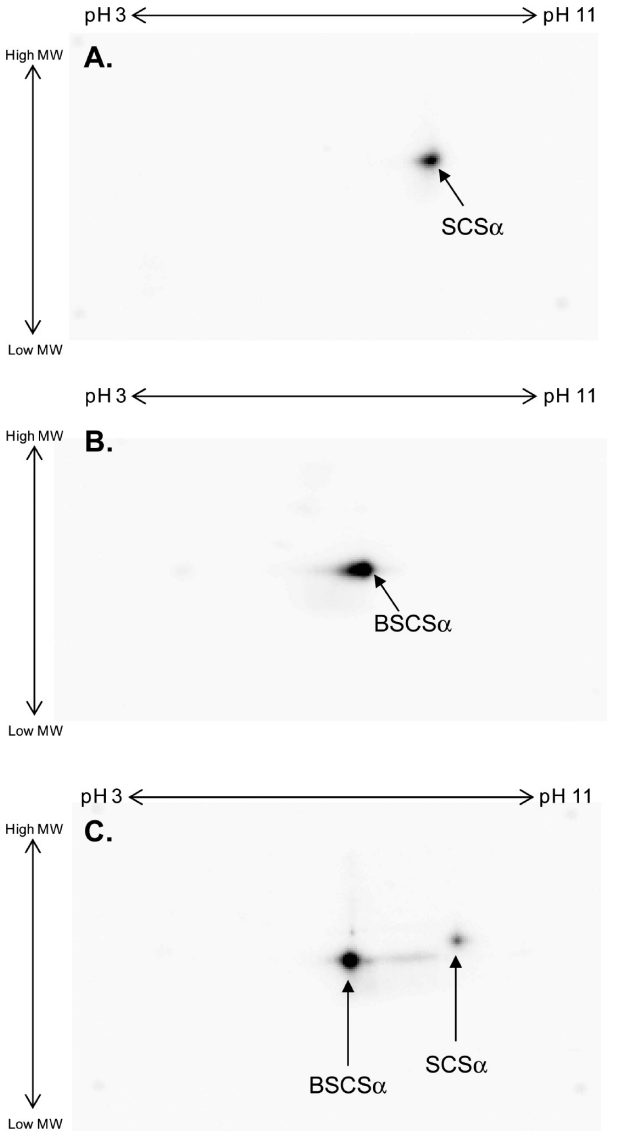

FIGURE 3: Confirmation of the phosphate-binding protein as succinyl-CoA synthetase. Panel A shows ^{32}P labeling of solubilized porcine heart mitochondria. Panel B shows ^{32}P labeling of purified bacterial succinyl-CoA synthetase. Panel C shows ^{32}P labeling of a mixture of solubilized porcine heart mitochondria and purified bacterial succinyl-CoA synthetase. Proteins were first separated by isoelectric focusing point, over a pH range of 3−11NL, and then by molecular mass from ~150 to 10 kDa. In all panels, an arrow is used to indicate the ^{32}P binding to succinyl-CoA synthetase following 2D SDS gel electrophoresis, where BSCSα represents the purified bacterial succinyl-CoA synthetase α-subunit and SCSα represents the porcine heart mitochondrial succinyl-CoA synthetase α-subunit.

^{32}P binding to SCSα was greater when analyzed with BN-PAGE compared to 2D SDS−BN-PAGE. Importantly, a strong ^{32}P association was also observed in the Complex V region of the BN-PAGE gel; however, unlike SCSα, the association of ^{32}P with Complex V was not detected following exposure to SDS via 2D BN-PAGE. This finding is consistent with the results described above and underlines the uniqueness of SCSα to remain noncovalently bound to ^{32}P throughout the SDS gel process. Next, ^{32}P binding to SCSα was tested for a pH sensitivity by precipitating ^{32}P-labeled purified BSCS under acidic (TCA) and neutral (methanol) extraction procedures. In the TCA-treated sample (Figure 5B), ^{32}P binding was significantly weaker than in the neutral-treated sample (Figure 5C). The pH sensitivity of this noncovalent binding of ^{32}P to SCSα is especially interesting

because it has been well-documented that SCSα has an acid labile histidine phosphorylation site (*10*−*14*). Taken together, these results highlight the difficulty of quantifying ^{32}P binding by SCSα after denaturing and bring into question the use of pH sensitivity alone in identifying histidine phosphorylation sites.

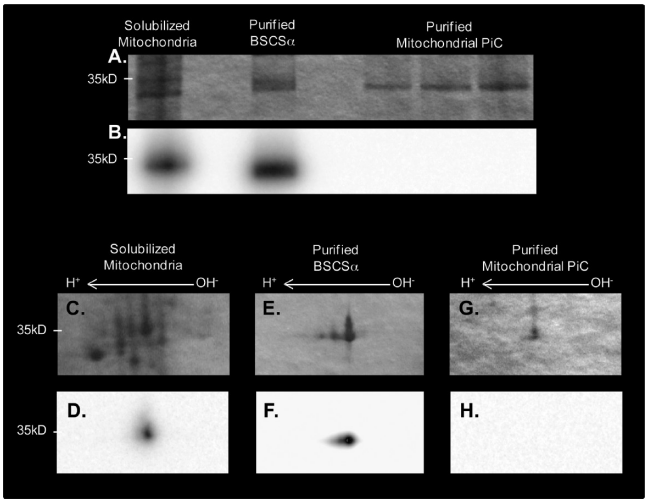

FIGURE 4: Coomassie blue-stained and ^{32}P-labeled solubilized mitochondria, purified succinyl-CoA synthetase, and purified mitochondrial phosphate-carrier protein. A 1D gel containing (from left to right) 25 μg of solubilized porcine heart mitochondria, 1 μg of purified bacterial succinyl-CoA synthetase, and 1, 2.5, and 5 μg of purified mitochondrial phosphate carrier stained with Coomassie blue (A) and labeled with ^{32}P (B). Panels C−H contain zoomed in regions of 2D gels containing 400 μg of solubilized porcine heart mitochondria (C and D), 20 mg of purified bacterial succinyl-CoA synthetase (E and F), and 25 μg of purified mitochondrial phosphate carrier (G and H). The top panel is stained with Coomassie blue, and the bottom panel is labeled with ^{32}P. For 2D gels, proteins were first separated by isoelectric focusing point, over a pH range of 3−11NL, and then by molecular mass from ~150 to 10 kDa; only the relevant region is shown. The purified bacterial succinyl-CoA synthetase α-subunit is abbreviated as BSCSα, and the purified mitochondrial phosphate-carrier protein is abbreviated as P_iC.

Effect of P_i on Succinyl-CoA Synthetase Activity. To determine the effect of binding of P_i on SCS's enzymatic activity, the reaction was run backward and the rate of succinate-dependent ATP hydrolysis was measured in both mitochondrial homogenates and purified BSCS. Since the SCS reaction can be run in both directions (*36*) and P_i is not a substrate for the reverse reaction, this assay enabled the effect of allosteric P_i binding to SCS to be determined. As shown in Figure 6, the addition of P_i increased SCS activity in a dose-dependent manner, both in protein homogenates and in BSCS. In mitochondrial homogenates, an initial increase in SCS activity was observed with the addition of 1 mM P_i and a maximal activity (more than 2-fold of control) was reached with 5 mM P_i (Figure 6A). Since the mitochondrial preparations used for this study included a P_i loading step, there was some concern that the dynamic range of SCS activity may be limited by a potentially high baseline P_i concentration. Thus, SCS activity was also measured in non-P_i-loaded mitochondria. However, no difference in SCS activity was observed between preparations, and therefore, these data were pooled. A less pronounced P_i-induced activation of SCS was also observed in purified BSCS (Figure 6B). It is important to note that the baseline P_i level and/or existence of small contaminants in this commercial preparation was unknown.

The P_i-induced activation of SCS was also determined with an in-gel assay, which measured the ATP and GTP hydrolytic capacity of BSCS (Figure 7). Using this in-gel assay approach, the P_i activation profile for BSCS more closely tracked that of porcine heart mitochondria (Figure 6A), compared to our in-solution studies described above. The results presented in Figure 7C also revealed that the ATP-dependent activity of BSCS was higher than the GTP-dependent activity, implying that the ATP-specific transcript may be more strongly expressed in this purified BSCS enzyme preparation. Since several studies have shown that the P_i and calcium effects for several mitochondrial proteins are additive (*26*, *30*), we also tested for a calcium-induced activation of SCS by incubating solubilized mitochondria and purified BSCS with 0 or 1 μM free calcium. Under our

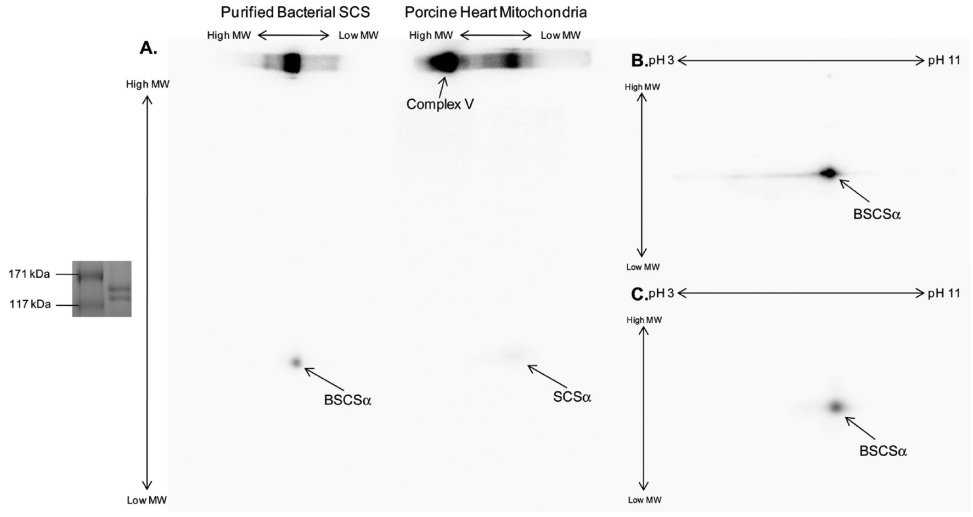

FIGURE 5: Effect of denaturation on the ^{32}P labeling of succinyl-CoA synthetase. After incubation with ^{32}P, succinyl-CoA synthetase was run using 1D and subsequent 2D BN-PAGE on purified succinyl-CoA synthetase or total porcine heart mitochondria (A) or was precipitated with methanol (B) or trichloroacetic acid (C). The insert to the left of panel A shows that the bacterial succinyl-CoA synthetase complex has a molecular mass of ~140 kDa, consistent with previous reports (*59*). Proteins were first separated by either molecular mass with BN-PAGE (A) or isoelectric focusing point (B and C) over a nonlinear pH range of 3−11 and then by molecular mass from ~150 to 10 kDa. In all panels, an arrow is used to indicate the ^{32}P binding to succinyl-CoA synthetase following SDS gel electrophoresis, where BSCSα represents the purified bacterial succinyl-CoA synthetase α-subunit and SCSα represents the porcine heart mitochondrial succinyl-CoA synthetase α-subunit.

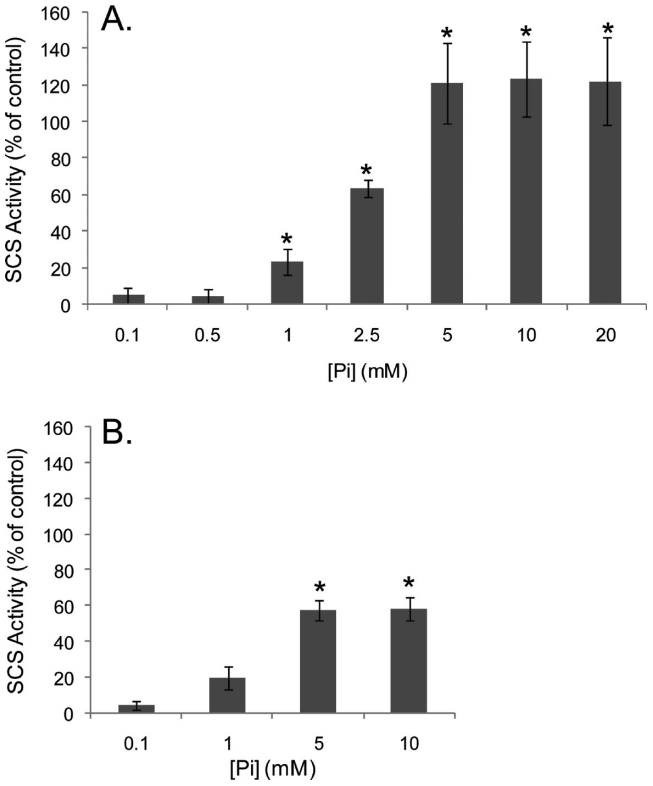

FIGURE 6: Effects of P_i on succinyl-CoA synthetase activity. Dose–response curves for the rate of succinate-dependent ATP hydrolysis in solubilized porcine heart mitochondria (A) and in purified bacterial succinyl-CoA synthetase (B) with various P_i concentrations, as measured in four experiments in porcine heart mitochondria and three experiments in purified protein. Activity is expressed as a percentage of the control (0 mM phosphate condition), where $p < 0.01$ is denoted with an asterisk.

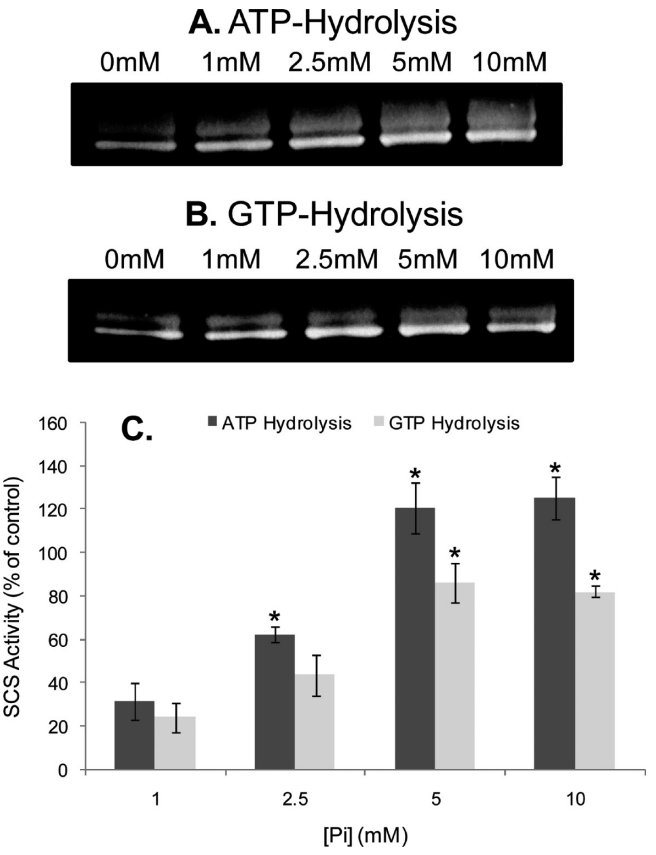

FIGURE 7: In-gel succinyl-CoA synthetase activity assay. Succinate-dependent ATP (A) and GTP (B) hydrolytic activities of purified bacterial succinyl-CoA synthetase pretreated with different concentrations of phosphate. The increase in succinyl-CoA synthetase activity with phosphate shown in panel C is quantified relative to the 0 mM phosphate condition across three experiments for each nucleotide. Activity is expressed as a percentage of the control (0 mM phosphate condition), where $p < 0.01$ is denoted with an asterisk.

experimental conditions, no calcium effect was observed for SCS (results not shown).

DISCUSSION

This study demonstrates that one of the sites of P_i regulation in mitochondrial metabolism is SCS. This was shown by monitoring the binding of ^{32}P to SCSα within the intact mitochondrial matrix (Figure 1), in solubilized mitochondrial homogenates (Figure 2), and in purified BSCS (Figure 3) as well as by measuring SCS activity in vitro (Figures 6 and 7). The binding of ^{32}P to SCSα in intact mitochondria was dependent on the mitochondrial energetic state, reflecting the concentration of matrix P_i. The ability of SCSα to bind ^{32}P in the absence of an energy source and under the harsh denaturing conditions of SDS revealed that ^{32}P labeling in 2D gel studies is not limited to covalent protein modifications. Additionally, the binding of P_i to SCS was associated with an activation of SCS activity both in mitochondrial homogenates and in purified BSCS. Taken together, these data reveal that the noncovalent binding of P_i to SCS is regulatory and linked to the energized state of the matrix. The pivotal role that SCS plays in the metabolism of the mitochondria is outlined in Figure 8. SCS plays a crucial role in the citric acid cycle, as the only site of substrate level phosphorylation within mitochondria, and as a component of ketone oxidation and heme synthesis.

It has previously been shown that P_i can directly activate citric acid cycle dehydrogenases, including α-ketoglutarate dehydrogenase (*26*), NAD-isocitrate dehydrogenase (*27*), and malate dehydrogenase (*28*), as well as several sites of oxidative

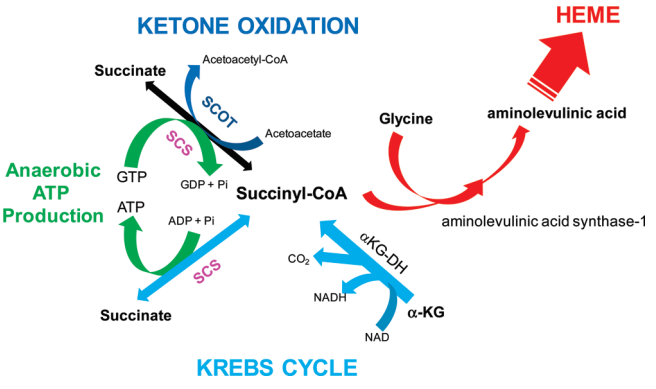

FIGURE 8: Role of succinyl-CoA synthetase in mitochondrial energetics and biosynthesis. Succinyl-CoA synthetase (SCS) is colored pink, and the pathways associated with the citric acid cycle are colored light blue. Ketone oxidation and succinyl-CoA acetoacetate CoA transferase (SCOT) are colored dark blue. Anaerobic ATP/GTP production is colored green and heme synthesis (i.e., porphyrin) red.

phosphorylation (*30*). This study demonstrates that P_i activates the maximum velocity of SCS in a dose-dependent manner in mitochondrial homogenates and purified BSCS protein. This allosteric effect of P_i is a classical V-type allosteric activation associated with many multisubunit enzymes, where the complexes are stabilized and both the forward and reverse maximum

velocities are increased by allosteric factors (*41*, *42*). Since P_i has been shown to stabilize the SCS complex (*8*) and increase the V_{max} of the reverse reaction, it is reasonable to assume that the forward reaction kinetics are also enhanced by P_i as found in other V-type interactions.

These results suggest that SCS is another target for P_i activation in mitochondrial energetics. Further support for a P_i-induced activation of SCS is found in the study by Siess et al. (*29*), who determined the effects of P_i on the matrix metabolite concentrations of α-ketoglutarate, succinyl-CoA, and malate in liver mitochondria. It was shown that P_i decreases matrix α-ketoglutarate and succinyl-CoA concentrations while increasing the malate concentration. This metabolite "crossover" is consistent, but not unique, to an activation of SCS by P_i (*29*). Also consistent with the activation of SCS in low-energy states is the ~2.5-fold increase in the level of the SCS product, succinate, during cardiac ischemia (*16*, *43*). Collectively, these results provide strong evidence that SCS activity is increased in the presence of millimolar P_i levels.

The P_i-induced activation of SCS is especially interesting in the functional context of myocardial ischemia or any energy supply and demand mismatch that occurs in the heart. The most rapid and large change in cytosolic metabolites that occurs during such an energetic mismatch in the heart involves the cytosolic P_i concentration (*44*). Thus, P_i is an excellent signaling molecule that is capable of activating the energy conversion processes of the mitochondria as well as potentially supporting non-oxygen-dependent substrate-level ATP formation. Under energy-limited conditions, such as myocardial ischemia or hypoxia, ATP production by anaerobic glycolysis can prevent broad cellular injury and maintain energization of the mitochondrial matrix by driving Complex V backward as an ATPase (*45*, *46*). However, during prolonged myocardial ischemia, anaerobic glycolysis is suppressed (*47*), and therefore, anaerobic pathways of mitochondrial metabolism (i.e., substrate-level phosphorylation via SCS) are triggered to support ATP production and potentially mollify cardiac injury (*16*, *17*, *43*, *48*). The increased activity of substrate-level phosphorylation via SCS has been suggested as the source of high succinate concentrations in the ischemic heart as discussed above. To support this notion, kidney proximal tubules subjected to hypoxia and reoxygenation demonstrated that anaerobic metabolism of α-ketoglutarate with aspartate increased the recovery of cellular ATP by intramitochondrial substrate-level phosphorylation at the level of SCS (*17*). Therefore, we speculate that P_i acts as both a substrate and an allosteric activator of substrate-level phosphorylation via SCS to generate matrix ATP that may limit ischemically induced mitochondrial dysfunction. We did not attempt to directly monitor the allosteric effects of P_i on matrix ATP at anoxia because the interpretation of such studies is complicated by the fact that P_i is also a substrate for the forward (ATP synthetic) reaction. Thus, unraveling the different effects of P_i as a substrate and/or allosteric modulator of SCS in intact mitochondria will be difficult.

Direct chemical activity measurements of P_i within the mitochondrial matrix of intact cells or even isolated mitochondria are not currently available. Since the pH gradient is small in heart mitochondria (*30*), it is likely that the driving force for the inner membrane transport of P_i is generated by a concentration gradient, where the matrix P_i concentration is much lower than the cytosolic P_i concentration. The cytosolic P_i concentration in the heart increases with oxygen delivery limitations, as discussed above, or near maximum work levels as detected by ^{31}P NMR (*49*). In skeletal muscle, the cytosolic P_i concentration increases more proportionally with workload but also exists at a considerably higher concentration than heart in the resting state (*50*). Thus, at present, the relationship between cytosolic P_i concentration and workload with P_i activity in the mitochondrial matrix is unknown. Furthermore, whether the P_i-induced activation process detected here is involved during normal work transitions in heart and skeletal muscle is also unknown.

Another unique finding of this study was that the noncovalent binding of ^{32}P to SCSα survived the entire SDS gel electrophoresis process. Notably, SCSα was the only mitochondrial protein in this study that bound ^{32}P in a manner that persisted throughout SDS gel electrophoresis. Given the harsh denaturing properties of SDS, it is generally assumed that noncovalent protein modifications do not persist. Thus, like previous reports (*51*), we initially assumed that ^{32}P labeling of SCSα resulted from phosphorylation. However, the ability to selectively dilute SCSα's ^{32}P incorporation with excess cold P_i, in the absence of a high-energy nucleotide, implied that a majority of SCSα's ^{32}P association resulted from simple P_i binding, and not phosphorylation. Furthermore, the labile nature of binding of ^{32}P to SCSα in the native protein (Figure 5), coupled with the inability to exchange out its ^{32}P after SDS gel electrophoresis, suggested that conformational changes to SCSα by SDS trap P_i within the protein. Predictably, this extremely tight, high-affinity binding of ^{32}P to SCSα allowed the label to persist throughout the SDS gel electrophoresis process.

This study also revealed a pH sensitivity for SCSα's ^{32}P binding, as acid treatment decreased the intensity of labeling. This is likely due to changes in the charge state of phosphate or the SCS binding site as a function of pH. Since mitochondria have a bacterial ancestry, several studies have used acid lability to screen for histidine phosphorylations in mitochondria. To date, SCSα is one of the few defined histidine phosphorylations in eukaryotes (*52*). Although the decrease in the level of ^{32}P labeling may result from alterations to the phosphate ion or conformational changes to SCSα, this pH sensitivity is important to consider when evaluating differences in the "phosphorylation" of SCSα, since such changes may also result from noncovalent P_i binding.

The apparent affinity for P_i in the activation of SCS was found to be in the millimolar range (Figures 6 and 7). However, P_i was found to bind SCSα in the ^{32}P gel studies despite the fact that only trace amounts of P_i were added to keep the specific activity of ^{32}P high. As described above, since binding of ^{32}P to SCSα persists throughout SDS gel electrophoresis, the interaction is a high-affinity one, which is inconsistent with the apparent millimolar affinity revealed in the activity assays. This apparent discrepancy is resolved by two observations. First, as shown in Figure 5A, only a small fraction of SCSα's ^{32}P labeling is resolved upon exposure to SDS in the 2D BN-PAGE studies. Consequently, even if the overall affinity for SCS is in the millimolar region, the high sensitivity of ^{32}P autoradiography detects only a small fraction of the associated P_i in the native protein. Additionally, we found that the ^{32}P could be exchanged out of the native protein but not the SDS-treated protein in the gel. These later data suggest that denaturing of the protein traps phosphate in a high-affinity site, thereby permitting its detection through the SDS−PAGE process. These observations imply that while in-gel ^{32}P binding studies reflect the association of P_i with SCSα, this approach cannot be used to determine the mole fraction of SCSα containing bound P_i.

This study reveals a specific noncovalent binding of ^{32}P to SCSα in substrate-depleted mitochondria. It is important to point out that the specific activity of ^{32}P in the matrix is predictably different in energized and substrate-depleted mitochondria. When mitochondria are energized with carbon substrates, the matrix phosphorylation potential is high, resulting in a low matrix P_i concentration and increasing the potential specific activity of ^{32}P in the matrix P_i pool, thereby increasing the sensitivity of ^{32}P binding. However, in the substrate-depleted matrix, the P_i concentration is predictably higher, resulting in a lower matrix ^{32}P specific activity, which decreases the ^{32}P binding sensitivity. Thus, the enhanced ^{32}P labeling of SCSα in substrate-depleted mitochondria occurred despite the predicted decrease in specific activity, underlining the fact that the strength of association of P_i with SCSα is increased in the energy-limited state.

The enhanced binding of ^{32}P to SCSα observed in the substrate-depleted matrix suggests that phosphate binding to SCS could be a marker of compromised energy conversion in mitochondria with a high matrix P_i concentration. Several ^{31}P NMR studies have described a large, NMR-invisible P_i pool in mitochondria [~60% (*53*)], believed to result from P_i that is bound to proteins or membranes (*53*−*58*). Could the binding of P_i to SCS contribute to this NMR invisible pool of P_i? Our 2D gels estimate SCSα to be at a concentration of 0.27 nmol/mg of mitochondria, by comparing the SCSα intensity with that of Complex IV. Assuming 2 μL of matrix volume per milligram, the concentration of SCS is on the order of 0.2 mM, which could be quite significant relative to the low matrix P_i concentration in the energized state (see above). The BN-PAGE analysis of the entire mitochondrial proteome also suggests that Complex V could be a significant binding site of matrix P_i under energized conditions (Figure 5A). However, in the energy-limited matrix, the P_i concentration is well above the SCS concentration, and SCS is therefore not likely to significantly influence the chemical activity of P_i. Thus, the binding of P_i by SCS under energy-limited conditions may primarily serve to activate substrate-level phosphorylation.

In summary, P_i was shown to bind and allosterically enhance SCS activity. In the context of energy supply/utilization mismatched conditions, such as ischemia and hypoxia, the findings presented here suggest that a P_i-induced activation of SCS may anaerobically generate ATP in the matrix to minimize mitochondrial dysfunction and enable cellular repair before the onset of irreversible injury. The role of this activation process during normal work transitions in heart and skeletal muscle is yet to be resolved.

ACKNOWLEDGMENT

We gratefully acknowledge Ilsa Rovira for her oversight of the radioisotope studies. We also thank Dr. Toren Finkel for the laboratory space.

SUPPORTING INFORMATION AVAILABLE

Mass spectrometry identification for the ^{32}P-labeled succinyl-CoA synthetase α-subunit. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Aponte, A. M., Phillips, D., Harris, R. A., Blinova, K., French, S., Johnson, D. T., and Balaban, R. S. (2009) ^{32}P labeling of protein phosphorylation and metabolite association in the mitochondria matrix. *Methods Enzymol. 457*, 63–80.
2. Aponte, A. M., Phillips, D., Hopper, R. K., Johnson, D. T., Harris, R. A., Blinova, K., Boja, E. S., French, S., and Balaban, R. S. (2009) Use of ^{32}P To Study Dynamics of the Mitochondrial Phosphoproteome. *J. Proteome Res. 8*, 2679–2695.
3. Bykova, N. V., Egsgaard, H., and Moller, I. M. (2003) Identification of 14 new phosphoproteins involved in important plant mitochondrial processes. *FEBS Lett. 540*, 141–146.
4. Hopper, R. K., Carroll, S., Aponte, A. M., Johnson, D. T., French, S., Shen, R. F., Witzmann, F. A., Harris, R. A., and Balaban, R. S. (2006) Mitochondrial matrix phosphoproteome: Effect of extra mitochondrial calcium. *Biochemistry 45*, 2524–2536.
5. Kaufman, S., Gilvarg, C., Cori, O., and Ochoa, S. (1953) Enzymatic oxidation of α-ketoglutarate and coupled phosphorylation. *J. Biol. Chem. 203*, 869–888.
6. Ottaway, J. H., McClellan, J. A., and Saunderson, C. L. (1981) Succinic thiokinase and metabolic control. *Int. J. Biochem. 13*, 401–410.
7. Labbe, R. F., Kurumada, T., and Onisawa, J. (1965) The role of succinyl-CoA synthetase in the control of heme biosynthesis. *Biochim. Biophys. Acta 111*, 403–415.
8. Fraser, M. E., James, M. N., Bridger, W. A., and Wolodko, W. T. (2000) Phosphorylated and dephosphorylated structures of pig heart, GTP-specific succinyl-CoA synthetase. *J. Mol. Biol. 299*, 1325–1339.
9. Johnson, J. D., Mehus, J. G., Tews, K., Milavetz, B. I., and Lambeth, D. O. (1998) Genetic evidence for the expression of ATP- and GTP-specific succinyl-CoA synthetases in multicellular eucaryotes. *J. Biol. Chem. 273*, 27580–27586.
10. Boyer, P. D., DeLuca, M., Ebner, K. E., Hultquist, D. E., and Peter, J. B. (1962) Identification of phospohistidine in digests from a probable intermediate of oxidative phosphorylation. *J. Biol. Chem. 237*, C3306–C3308.
11. Bridger, W. A. (1971) Evidence for two types of subunits in succinyl coenzyme A synthetase. *Biochem. Biophys. Res. Commun. 42*, 948–954.
12. Leitzmann, C., Wu, J. Y., and Boyer, P. D. (1970) Subunits, composition, and related properties of succinyl coenzyme A synthetase. *Biochemistry 9*, 2338–2346.
13. Mitchell, R. A., Butler, L. G., and Boyer, P. D. (1964) The association of readily-soluble bound phosphohistidine from mitochondria with succinate thiokinase. *Biochem. Biophys. Res. Commun. 16*, 545–550.
14. Steiner, A. W., and Smith, R. A. (1981) Endogenous protein phosphorylation in rat brain mitochondria: Occurrence of a novel ATP-dependent form of the autophosphorylated enzyme succinyl-CoA synthetase. *J. Neurochem. 37*, 582–593.
15. Holt, S. J., and Riddle, D. L. (2003) SAGE surveys *C. elegans* carbohydrate metabolism: Evidence for an anaerobic shift in the long-lived dauer larva. *Mech. Ageing Dev. 124*, 779–800.
16. Pisarenko, O. I., Solomatina, E. S., Ivanov, V. E., Studneva, I. M., Kapelko, V. I., and Smirnov, V. N. (1985) On the mechanism of enhanced ATP formation in hypoxic myocardium caused by glutamic acid. *Basic Res. Cardiol. 80*, 126–134.
17. Weinberg, J. M., Venkatachalam, M. A., Roeser, N. F., and Nissim, I. (2000) Mitochondrial dysfunction during hypoxia/reoxygenation and its correction by anaerobic metabolism of citric acid cycle intermediates. *Proc. Natl. Acad. Sci. U.S.A. 97*, 2826–2831.
18. Garlid, K. D. (1980) On the mechanism of regulation of the mitochondrial K^+/H^+ exchanger. *J. Biol. Chem. 255*, 11273–11279.
19. Gunter, T. E., Wingrove, D. E., Banerjee, S., and Gunter, K. K. (1988) Mechanisms of mitochondrial calcium transport. *Adv. Exp. Med. Biol. 232*, 1–14.
20. Jung, D. W., Apel, L., and Brierley, G. P. (1990) Matrix free Mg^{2+} changes with metabolic state in isolated heart mitochondria. *Biochemistry 29*, 4121–4128.
21. Halestrap, A. P. (1994) Regulation of mitochondrial metabolism through changes in matrix volume. *Biochem. Soc. Trans. 22*, 522–529.
22. Izzard, S., and Tedeschi, H. (1970) Ion transport underlying metabolically controlled volume changes of isolated mitochondria. *Proc. Natl. Acad. Sci. U.S.A. 67*, 702–709.
23. Izzard, S., and Tedeschi, H. (1973) Characterization of orthophosphate-induced active cation transport in isolated liver mitochondria. *Arch. Biochem. Biophys. 154*, 527–539.
24. Kushnareva, Y. E., Haley, L. M., and Sokolove, P. M. (1999) The role of low ($<$ or $=$ 1 mM) phosphate concentrations in regulation of mitochondrial permeability: Modulation of matrix free Ca^{2+} concentration. *Arch. Biochem. Biophys. 363*, 155–162.
25. Basso, E., Petronilli, V., Forte, M. A., and Bernardi, P. (2008) Phosphate is essential for inhibition of the mitochondrial permeability transition pore by cyclosporin A and by cyclophilin D ablation. *J. Biol. Chem. 283*, 26307–26311.

26. Rodriguez-Zavala, J. S., Pardo, J. P., and Moreno-Sanchez, R. (2000) Modulation of 2-oxoglutarate dehydrogenase complex by inorganic phosphate, Mg^{2+}, and other effectors. *Arch. Biochem. Biophys. 379*, 78–84.
27. Hansford, R. G. (1972) Some properties of pyruvate and 2-oxoglutarate oxidation by blowfly flight-muscle mitochondria. *Biochem. J. 127*, 271–283.
28. Blonde, D. J., Kresack, E. J., and Kosicki, G. W. (1967) The effects of ions and freeze-thawing on supernatant and mitochondrial malate dehydrogenase. *Can. J. Biochem. 45*, 641–650.
29. Siess, E. A., Kientsch-Engel, R. I., Fahimi, F. M., and Wieland, O. H. (1984) Possible role of P_i supply in mitochondrial actions of glucagon. *Eur. J. Biochem. 141*, 543–548.
30. Bose, S., French, S., Evans, F. J., Joubert, F., and Balaban, R. S. (2003) Metabolic network control of oxidative phosphorylation: Multiple roles of inorganic phosphate. *J. Biol. Chem. 278*, 39155–39165.
31. Territo, P. R., French, S. A., Dunleavy, M. C., Evans, F. J., and Balaban, R. S. (2001) Calcium activation of heart mitochondrial oxidative phosphorylation: Rapid kinetics of mVO2, NADH, and light scattering. *J. Biol. Chem. 276*, 2586–2599.
32. Wolodko, W. T., Fraser, M. E., James, M. N., and Bridger, W. A. (1994) The crystal structure of succinyl-CoA synthetase from *Escherichia coli* at 2.5-Å resolution. *J. Biol. Chem. 269*, 10883–10890.
33. Wohlrab, H. (1980) Purification of a reconstitutively active mitochondrial phosphate transport protein. *J. Biol. Chem. 255*, 8170–8173.
34. Blinova, K., Levine, R. L., Boja, E. S., Griffiths, G. L., Shi, Z. D., Ruddy, B., and Balaban, R. S. (2008) Mitochondrial NADH fluorescence is enhanced by complex I binding. *Biochemistry 47*, 9636–9645.
35. Hellman, U., Wernstedt, C., Gonez, J., and Heldin, C. H. (1995) Improvement of an "In-Gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. *Anal. Biochem. 224*, 451–455.
36. Cha, S., and Parks, R. E. Jr. (1964) Succinic thiokinase. I. Purification of the enzyme from pig heart. *J. Biol. Chem. 239*, 1961–1967.
37. Schagger, H., and von Jagow, G. (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem. 199*, 223–231.
38. Stappen, R., and Kramer, R. (1993) Functional properties of the reconstituted phosphate carrier from bovine heart mitochondria: Evidence for asymmetric orientation and characterization of three different transport modes. *Biochim. Biophys. Acta 1149*, 40–48.
39. Stappen, R., and Kramer, R. (1994) Kinetic mechanism of phosphate/phosphate and phosphate/OH^- antiports catalyzed by reconstituted phosphate carrier from beef heart mitochondria. *J. Biol. Chem. 269*, 11240–11246.
40. Wohlrab, H., and Flowers, N. (1982) pH gradient-dependent phosphate transport catalyzed by the purified mitochondrial phosphate transport protein. *J. Biol. Chem. 257*, 28–31.
41. Lara-Lemus, R., and Calcagno, M. L. (1998) Glucosamine-6-phosphate deaminase from beef kidney is an allosteric system of the V-type. *Biochim. Biophys. Acta 1388*, 1–9.
42. Monod, J., Wyman, J., and Changeux, J. P. (1965) On the nature of allosteric transitions: A plausible model. *J. Mol. Biol. 12*, 88–118.
43. Pisarenko, O. I. (1996) Mechanisms of myocardial protection by amino acids: Facts and hypotheses. *Clin. Exp. Pharmacol. Physiol. 23*, 627–633.
44. Balaban, R. S. (2009) Domestication of the cardiac mitochondrion for energy conversion. *J. Mol. Cell. Cardiol.* h (in press).
45. Nieminen, A. L., Saylor, A. K., Tesfai, S. A., Herman, B., and Lemasters, J. J. (1995) Contribution of the mitochondrial permeability transition to lethal injury after exposure of hepatocytes to t-butylhydroperoxide. *Biochem. J. 307* (1), 99–106.
46. Simbula, G., Glascott, P. A. Jr., Akita, S., Hoek, J. B., and Farber, J. L. (1997) Two mechanisms by which ATP depletion potentiates induction of the mitochondrial permeability transition. *Am. J. Physiol. 273*, C479–C488.
47. Neely, J. R., and Grotyohann, L. W. (1984) Role of glycolytic products in damage to ischemic myocardium. Dissociation of adenosine triphosphate levels and recovery of function of reperfused ischemic hearts. *Circ. Res. 55*, 816–824.
48. Sanadi, D. R., and Fluharty, A. L. (1963) On the mechanism of oxidative phosphorylation. VII. The energy-requiring reduction of pyridine nucleotide by succinate and the energy-yielding oxidation of reduced pyridine nucleotide by fumarate. *Biochemistry 2*, 523–528.
49. Gard, J. K., Kichura, G. M., Ackerman, J. J., Eisenberg, J. D., Billadello, J. J., Sobel, B. E., and Gross, R. W. (1985) Quantitative ^{31}P nuclear magnetic resonance analysis of metabolite concentrations in Langendorff-perfused rabbit hearts. *Biophys. J. 48*, 803–813.
50. Vicini, P., and Kushmerick, M. J. (2000) Cellular energetics analysis by a mathematical model of energy balance: Estimation of parameters in human skeletal muscle. *Am. J. Physiol. 279*, C213–C224.
51. Ferrari, S., Moret, V., and Siliprandi, N. (1990) Protein phosphorylation in rat liver mitochondria. *Mol. Cell. Biochem. 97*, 9–16.
52. Puttick, J., Baker, E. N., and Delbaere, L. T. (2008) Histidine phosphorylation in biological systems. *Biochim. Biophys. Acta 1784*, 100–105.
53. Humphrey, S. M., and Garlick, P. B. (1991) NMR-visible ATP and P_i in normoxic and reperfused rat hearts: A quantitative study. *Am. J. Physiol. 260*, H6–H12.
54. Adler, S., Shoubridge, E., and Radda, G. K. (1984) Estimation of cellular pH gradients with ^{31}P-NMR in intact rabbit renal tubular cells. *Am. J. Physiol. 247*, C188–C196.
55. Arnold, D. L., Matthews, P. M., and Radda, G. K. (1984) Metabolic recovery after exercise and the assessment of mitochondrial function in vivo in human skeletal muscle by means of ^{31}P NMR. *Magn. Reson. Med. 1*, 307–315.
56. Bailey, I. A., Williams, S. R., Radda, G. K., and Gadian, D. G. (1981) Activity of phosphorylase in total global ischaemia in the rat heart. A phosphorus-31 nuclear-magnetic-resonance study. *Biochem. J. 196*, 171–178.
57. Eriksson, O., Pollesello, P., and Saris, N. E. (1994) Effect of phenylephrine on the compartmentation of inorganic phosphate in perfused rat liver during gluconeogenesis and urea synthesis: A ^{31}P-NMR-spectroscopic study. *Biochem. J. 298* (1), 17–21.
58. Hutson, S. M., Williams, G. D., Berkich, D. A., LaNoue, K. F., and Briggs, R. W. (1992) A ^{31}P NMR study of mitochondrial inorganic phosphate visibility: effects of Ca^{2+}, Mn^{2+}, and the pH gradient. *Biochemistry 31*, 1322–1330.
59. Buck, D., Spencer, M. E., and Guest, J. R. (1985) Primary structure of the succinyl-CoA synthetase of *Escherichia coli*. *Biochemistry 24*, 6245–6252.