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Intrinsic Protein Kinase Activity in Mitochondrial Oxidative Phosphorylation Complexes

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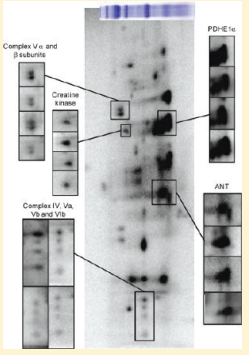
Supporting Information

ABSTRACT: Mitochondrial protein phosphorylation is a well-recognized metabolic control mechanism, with the classical example of pyruvate dehydrogenase (PDH) regulation by specific kinases and phosphatases of bacterial origin. However, despite the growing number of reported mitochondrial phosphoproteins, the identity of the protein kinases mediating these phosphorylation events remains largely unknown. The detection of mitochondrial protein kinases is complicated by the low concentration of kinase relative to that of the target protein, the lack of specific antibodies, and contamination from associated, but nonmatrix, proteins. In this study, we use blue native gel electrophoresis (BN-PAGE) to isolate rat and porcine heart mitochondrial complexes for screening of protein kinase activity. To detect kinase activity, one-dimensional BN-PAGE gels were exposed to [γ-^{32}P]ATP and then followed by sodium dodecyl sulfate gel electrophoresis. Dozens of mitochondrial proteins were labeled with ^{32}P in this setting, including all five complexes of oxidative phosphorylation and several citric acid cycle enzymes. The nearly ubiquitous ^{32}P protein labeling demonstrates protein kinase activity within each mitochondrial protein complex. The validity of this two-dimensional BN-PAGE method was demonstrated by detecting the known PDH kinases and phosphatases within the PDH complex band using Western blots and mass spectrometry. Surprisingly, these same approaches detected only a few additional conventional protein kinases, suggesting a major role for autophosphorylation in mitochondrial proteins. Studies on purified Complex V and creatine kinase confirmed that these proteins undergo autophosphorylation and, to a lesser degree, tenacious ^{32}P−metabolite association. In-gel Complex IV activity was shown to be inhibited by ATP, and partially reversed by phosphatase activity, consistent with an inhibitory role for protein phosphorylation in this complex. Collectively, this study proposes that many of the mitochondrial complexes contain an autophosphorylation mechanism, which may play a functional role in the regulation of these multiprotein units.



Mitochondria are dynamically modified organelles that perform several vital functions in the eukaryotic cell. In addition to their role in energy metabolism, mitochondria are also involved in intermediary metabolism, biochemical synthesis, calcium signaling, redox regulation, and apoptosis.[1,2] The complexity of mitochondria requires a sophisticated system of intracellular communication, capable of responding rapidly to changes in cellular energy metabolism as well as many other processes. In recent years, reversible protein phosphorylation has emerged as a potential ubiquitous regulatory mechanism in mitochondria. Several proteomic screening studies, using a combination of sodium dodecyl sulfate (SDS) gel electrophoresis and mass spectrometry, have revealed an extensive network of phosphoproteins in mitochondria.[3−10] Additionally, our lab recently coupled ^{32}P labeling in intact mitochondria with two-dimensional (2D) gel electrophoresis to identify dozens of mitochondrial proteins with potential regulatory (i.e., ^{32}P turnover) phosphorylation sites.[3,5] While data for the functionality of the mitochondrial phosphoproteome continue to accumulate,[5,11,12] the exact mechanisms governing reversible phosphorylation remain poorly defined for a majority of mitochondrial proteins. In fact, unlike pyruvate dehydrogenase (PDH) and branched chain α-ketoacid dehydrogenase (BCKDH) complexes, which have their own matrix-localized kinases,[13,14] most mitochondrial phosphoproteins have not been linked to specific kinases. To date, no experimental studies have directly examined the localization of active kinases to mitochondrial protein complexes outside of the PDH complex. Thus, identifying the matrix kinases remains an important challenge for resolving the mechanism and regulation of mitochondrial phosphorylation events.

The relatively low abundance of mitochondrial kinases in complex mixtures limits the usefulness of mass spectrometry and gel electrophoresis approaches for detecting these proteins. That is, the ratio of target protein to kinase is on the order of 10−100 or even higher, resulting in a very low concentration of kinase protein. Furthermore, current techniques are unable to distinguish mitochondrial kinases from copurifying contaminants (i.e., cytosolic proteins associated with the mitochondrial outer membrane). This is especially problematical when a kinase is present in both the cytosol and mitochondrial matrix, as this makes enhancement via mitochondrial purification difficult to interpret.[15,16] For instance,

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more than ~30% of the heart's cellular protein is mitochondrial, which means that the enhancement ratio can only approach 4 under the best of conditions.

To better screen for active mitochondrial kinases, we coupled direct ATP-dependent ^{32}P labeling in isolated protein complexes with blue native gel electrophoresis (BN-PAGE). Specifically, the nondenaturing conditions of BN-PAGE were used to resolve mitochondrial enzyme complexes in their near native conformation[17] in the pig and rat heart mitochondria. To screen for protein phosphorylation events, we incubated these mitochondrial complexes in vitro with [γ-^{32}P]ATP and resolved them into individual protein subunits by SDS gel electrophoresis (2D BN/SDS-PAGE). Autoradiography of the 2D BN/SDS-PAGE gels directly evaluated protein phosphorylation and other covalent modifications, because the presence of SDS removed a majority of weak metabolite associations.

This study revealed dozens of kinase-mediated phosphorylations for the proteins associated with all five complexes of oxidative phosphorylation. However, despite this extensive network of mitochondrial protein phosphorylation, few kinases—with the exception of the well-characterized mitochondrial PDH kinases—were detected. These data suggest a significant role for autophosphorylation within the mitochondrial oxidative phosphorylation protein complexes.

MATERIALS AND METHODS

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

Mitochondrial Isolation and Incubation Conditions. 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 National Heart, Lung and Blood Institute Animal Care and Use Committee. Porcine heart mitochondria were isolated from tissue that was cold-perfused in situ to remove blood and extracellular calcium as well as to prevent any warm ischemia, as previously described.[3] Isolated rat heart mitochondria were prepared under identical conditions. Mitochondrial preparations were tested for viability by measuring both the respiratory control ratio (RCR) (>8-fold) and the ability to maintain matrix ATP (>2 nmol/mg of mitochondrial protein) content, as previously described.[3] Protein concentrations were determined using a Bradford assay (USB quantification reagent, USB, Cleveland, OH), against a standard curve of BSA.

Our previous study revealed strong incorporation of ^{32}P for several mitochondrial proteins that were dephosphorylated during isolation as a means of restoring their native, steady-state phosphorylation status.[3] Because this study sought to screen for kinase-mediated ^{32}P turnover, the mitochondrial protein phosphate pools were rebuilt by incubating mitochondria (1 mg/mL) in oxygen-saturated buffer A [125 mM KCl, 15 mM NaCl, 20 mM HEPES, 5 mM $MgCl_2$, 1 mM K_2EDTA, and 1 mM K_2EGTA (pH 7.1)] containing 5 mM potassium glutamate, 5 mM potassium malate, and 5 mM inorganic phosphate at 37 °C for 20 min. To ensure adequate oxygen for oxidative phosphorylation, 100% oxygen was passed over the incubation medium. Immediately following incubation, mitochondria were centrifuged at 10000 rpm for 5 min at 4 °C, and the pellet was used for BN-PAGE.

In Vitro ^{32}P Labeling of Mitochondria Separated by 2D BN/SDS-PAGE. BN-PAGE was used to maintain mitochondrial protein complexes in their intact form.[17] BN-PAGE relies on the binding of Coomassie Blue to the protein complexes to enhance the differential migration of the protein complexes during the PAGE process. BN-PAGE was performed according to the Invitrogen protocol for the NativePAGE Novex Bis-Tris Gel system,[18] with dodecyl maltoside as the solubilizing detergent. Bis-Tris gels (4 to 16%, 1 mm) were used, with 75 μg of mitochondrial protein loaded per well. Electrophoresis was performed at 4 °C and 150 V for 1 h and then at 250 V for 1.3 h.

Immediately following electrophoresis, BN-PAGE gel slices were cut and incubated with buffer A for 10 min at room temperature to remove excess Coomassie Blue. Each BN-PAGE gel slice was then placed in a 15 mL tube, containing 3.5 mL of buffer A and 350 μCi of [α-^{32}P]ATP, [γ-^{32}P]ATP, [γ-^{32}P]GTP, or ^{32}P. We reasoned that keeping the level of radioactivity was a better normalizing factor than concentration because the specific activities of the metabolites were close to each other. The absolute activity used was primarily based on radiation safety concerns. On the basis of the specific activity of these metabolites, the final concentrations of each metabolite were as follows: 33 μM [α-^{32}P]ATP, 33 μM [γ-^{32}P]ATP, 17 μM [γ-^{32}P]GTP, or 11 μM ^{32}P. BN-PAGE lanes were incubated for 4.5 h at room temperature, with agitation, and then washed in buffer A four times, for 15 min each to remove the exogenous metabolite. BN-PAGE gel slices were incubated for 5 min in 5 mL of buffer A, first containing 50 mg of DTT and then 125 mg of iodoacetemide. Each BN-PAGE gel slice was rinsed briefly with 1× TGS buffer [25 mM Tris (pH 8.3), 192 mM glycine, and 0.1% SDS] and applied to a 10 to 15% SDS gel (Nextgen Sciences, Ann Arbor, MI) and sealed with 1% agarose, containing bromophenol blue. Electrophoresis was performed in an Ettan DALT-12 tank (GE Healthcare) at 20 °C in electrophoresis buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, and 0.2% SDS for ~2100 V h. After electrophoresis, 2D BN-PAGE gels were stained with Coomassie Blue, containing 50% methanol, 3% phosphoric acid, and 0.075% (w/v) Coomassie Blue G-250 (Bio-Rad Laboratories, Hercules, CA) for 2 h. Gels were destained for 30 min with 50% methanol and 3% phosphoric acid, followed by 1.5 h with 30% methanol and 3% phosphoric acid. Radiolabeled gels were briefly rehydrated with deionized water, placed on filter paper, and dried in a large format dryer (Bio-Rad), before being exposed to a phosphor screen (GE Healthcare) for 72 h and scanned on a Typhoon 9410 imager (GE Healthcare), as previously described.[3] Non radiolabeled gels were scanned using the Typhoon 9400 scanner at an excitation wavelength of 532 nm, with a 560LP emission filter.

Mass Spectrometry Identifications. Protein bands from one-dimensional (1D) BN-PAGE gels were excised and destained overnight in 50% MeOH and 50 mM NH_4HCO_3. In-gel digestion was performed as previously described using trypsin or chymotrypsin.[19] Peptides were extracted using a 50:50:0.1 (v/v/v) water/ACN/HCOOH mixture in a water bath sonicator (25 °C, 10 min), three times. The desalted digests were analyzed via LC-MS/MS with an LTQ-Orbitrap Velos (Thermo-Fisher Scientific) interfaced with an Eksigent (Dublin, CA) nanoLC-Ultra 1D plus system. Peptides were first loaded onto a Zorbax 300SB-C18 trap column (Agilent, Palo Alto, CA) at a flow rate of

6 μL/min for 6 min and then separated on a reversed-phase PicoFrit analytical column (New Objective, Woburn, MA) using a 90 min linear gradient from 5 to 40% acetonitrile in 0.1% formic acid at a flow rate of 250 nL/min. LTQ-Orbitrap Velos settings were as follows: spray voltage of 1.5 kV and full MS mass range from m/z 230 to 2000. The LTQ-Orbitrap Velos was operated in a data-dependent mode [i.e., one MS1 high-resolution (60000) scan for precursor ions followed by six data-dependent MS2 scans for precursor ions above a threshold ion count of 2000 with a collision energy of 35%].

To obtain protein identifications from 2D BN/SDS-PAGE gels, we excised protein spots, washed them, digested them with trypsin (37 °C, 4 h) using an Ettan Spot Handling Workstation (GE Healthcare), and identified them with the LTQ-Orbitrap Velos with the same settings as described above except the linear gradient was 20 min. To assign protein identifications to the radiolabeled 2D BN/SDS-PAGE gels, radiolabeled and nonradiolabeled gels were aligned, as previously described.[3]

All files generated from the LTQ-Orbitrap Velos (.RAW files) were processed with Proteome Discoverer, version 1.1 (Thermo-Fisher Scientific), prior to being submitted to our six-processor Mascot cluster at the National Institutes of Health (http://biospec.nih.gov, version 2.3) using the following criteria: database, SwissProt; taxonomy, human, pig, and bovine; enzyme, trypsin; number of miscleavages, two; fixed modifications, carbamidomethylation (+57 Da); variable modifications, methionine oxidation (+16 Da); acetyl (Prot N-term) and deamidation (NQ), precursor tolerance set to 15 ppm; MS/MS tolerance set to 0.8 Da. The combined pig and human databases and bovine databases were used in this case because porcine protein sequences are not fully available at present. For the rat heart mitochondria, the rat database was used exclusively. The automatic decoy database search option was selected, and only the high-confidence (FDR, 0.01) peptides were accepted for protein identification. Briefly, every time a peptide sequence search is performed on a target database, a random sequence of equal length is automatically generated and tested. The statistics for matches are calculated, and a peptide significance is generated; an in-depth explanation can be found at the Matrix Science website (http://www.matrixscience.com).

Targeted Mass Spectrometry Kinase/Phosphatase Screen. To screen for kinases and phosphatases from both pig and rat, 10 bands each of mitochondrial Complexes I−V and PDH were extracted from BN-PAGE lanes, pooled, and digested with trypsin, and another set was treated with chymotrypsin. The complexes were prepared for mass spectrometry as described above. Liquid chromatography and tandem mass spectrometry were then performed using an Eksigent nanoLC-Ultra 1D plus system coupled to an LTQ Orbitrap Velos mass spectrometer (Thermo-Fisher Scientific, San Jose, CA) using CID fragmentation. Peptides were first loaded onto a Zorbax 300SB-C18 trap column (Agilent) at a flow rate of 6 μL/min for 6 min and then separated on a reversed-phase PicoFrit analytical column (New Objective) using a 90 min linear gradient from 5 to 40% acetonitrile in 0.1% formic acid at a flow rate of 250 nL/min. LTQ-Orbitrap Velos settings were as follows: spray voltage of 1.5 kV and full MS mass range from m/z 230 to 2000. The LTQ-Orbitrap Velos was operated in a data-dependent mode [i.e., one MS1 high-resolution (60000) scan for precursor ions followed by six data-dependent MS2 scans for precursor ions above a threshold ion count of 2000 with a collision energy of 35%]. To generate complementary fragment ions, we used the higher-energy collision dissociation (HCD) cell to yield different fragmentation patterns and to achieve more identifications. The settings for HCD were the same except for the MS1 resolution (30000) scan for precursor ions and the collision energy was set to 45% and the MS2 resolution to 7500. The raw files generated were processed as described above except for the chymotrypsin digest samples. The enzyme option was set to chymotrypsin, and when the HCD mode was used, the MS/MS tolerance was set to 0.1 Da.

Repository for Data. All positively identified proteins were deposited into Tranche data repository and be found in the Proteome Commons public proteomics database. A positive protein identification consists of the following criteria: (1) two or more unique peptides with a Mascot peptide rank of 1 and an IonScore above the threshold set for high confidence (FDR ≤ 0.01) or one unique peptide that was sampled more then once with the same scoring criteria described above. The public repository can be found at https://proteomecommons.org/group.jsp?i=219. These data include all of the proteins identified in the 1D BN-PAGE and 1D BN/SDS PAGE gels of the rat and pig.

Western Analysis. Western blots were performed on porcine mitochondrial proteins separated by 2D BN/SDS-PAGE, as described above with the following modifications. BN-PAGE gel slices were applied to 8 to 16% SDS gels (Bio-Rad) and run in a Criterion Cell (Bio-Rad) for ∼210 V h. Proteins were blotted to a nitrocellulose membrane, and pyruvate dehydrogenase kinase 1 (PDK1) was detected with anti-PDK1 (Stressgen, Ann Arbor, MI) as the primary antibody and Alexa Fluor 488 anti-rabbit IgG (Invitrogen) as the secondary antibody. Blots were visualized using a Typhoon 9400 imager (GE Healthcare). In addition to PDK1, several other kinases were screened, including PDK2, PDK3, PDK4 (see below), JAK1, JAK2, PKCβ1, PKCδ, and PKCε (Santa Cruz Biotechnology, Santa Cruz, CA), PKA Cα and MEK1 (Cell Signaling Technology, Danvers, MA), and MEK6 (Genway Bio, San Diego, CA). Purified kinases were obtained from Invitrogen for MEK6, PKCδ, and PKCε, to further validate the antibodies. For studies probing 2D BN/SDS-PAGE gels alongside purified proteins and isolated mitochondria, 0.05 μg of protein and 100 μg of mitochondria were loaded. When probing for PDK4, we initially used an antibody from Abgent (San Diego, CA, catalog no. AP7041b). After conducting several experiments (Figure S3 of the Supporting Information), we believe that the dominant interaction of this PDK4 antibody in mitochondrial proteins is with the α subunit of Complex V and recommend caution for any laboratories using this antibody to probe for PDK4 in mitochondria.

Autocatalytic Reactions in Purified Proteins. To screen for autocatalytic processes, 100 μg of Complex V was purified via immunocapture (Mitosciences, Eugene, OR) and incubated in enzyme buffer [60 mM sucrose, 50 mM Tris-HCl, 50 mM KCl, 4 mM $MgCl_2$, and 2 mM EGTA (pH 8.0)] at a concentration of 0.5 μg/μL for 5 min at 37 °C. The enzymatic reaction was started via addition of a mixture of 2 mM ATP and 150 μCi of [α-^{32}P]ATP or [γ-^{32}P]ATP and incubation for an additional 30 min at 37 °C. Cold ATP was added for two reasons. ATP was necessary (1) to achieve proper isoelectric focusing of Complex V and (2) to approach the specific activity of [^{32}P]ATP in the matrix of intact mitochondria. The reaction was stopped via addition of 50 μL of lysis buffer [15 mM Tris-HCl, 7 M urea, 2 M thiourea, and 4% CHAPS (w/v) (pH 8.5)] and placement of the sample on ice for 15 min. ^{32}P-labeled Complex V was then analyzed by 2D gel electrophoresis, as previously described.[3]

Creatine kinase (CK) was also screened for autocatalytic processes using the method described above, with slight modifications. Ten micrograms of purified CK (Sigma-Aldrich, St. Louis, MO; catalog no. C9858) was incubated in buffer B [100 mM glycylglycine, 2 mM $MgCl_2$, 0.5 mM EDTA, and 2 mM DTT (pH 8.4); adapted from ref 20] at a concentration of 1 μg/μL for 5 min at 37 °C. The reaction was started by addition of a mixture of 2 mM ATP and 50 μCi of [α-^{32}P]ATP or [γ-^{32}P]ATP and incubation for an additional 30 min at 37 °C. The reaction was stopped by addition of 50 μL of lysis buffer and placement of the sample on ice for 15 min. ^{32}P-labeled CK was then analyzed by 2D gel electrophoresis, as described above.

Complex IV Activity. To determine if ATP altered enzyme activity, gel strips were incubated for 1 h at room temperature in preincubation buffer [125 mM KCl, 15 mM NaCl, 20 mM HEPES, 1 mM EGTA, 1 mM EDTA, and 5 mM $MgCl_2$ (pH 7.1)] with 0−5 mM ATP. Complex IV activity was then measured for 30 min using an in-gel assay, as previously described.[21] To screen for a recovery of Complex IV activity, gel strips were incubated with 0 or 5 mM ATP for 2 h or with 5 mM ATP for 1 h and then with 0 mM ATP or protein phosphatase 1 (PP1) (Sigma) for the subsequent hour.

The linearity of this assay was conducted over protein concentrations of 2.5−75 μg of mitochondrial protein per lane as well as from 15 to 60 min. The assay was linear over all of these concentration and temporal ranges (see Figure S1 of the Supporting Information).

Statistical Analysis. Data are reported as means ± the standard deviation and were analyzed using a Student's t test. A p value of <0.01 was considered statistically significant and is denoted with an asterisk.

RESULTS

Protein Identification for One- and Two-Dimensional BN-PAGE. BN-PAGE relies on the separation of intact mitochondrial protein complexes in the first dimension, and 2D BN/SDS-PAGE relies on the separation of the complexes into their individual subunits via SDS gel electrophoresis. In both dimensions, separation is primarily dependent on the molecular mass of the complexes and individual proteins, respectively. A key advantage of 2D BN/SDS-PAGE is that it provides information about protein interactions and the multienzymatic processes (i.e., post-translational modifications) that occur at a natural level of cellular compartmentalization.[22] Figure 1 shows the dominant mitochondrial protein complexes in each 1D BN-PAGE band and provides identifications for the individual protein subunits separated via 2D BN/SDS-PAGE in the pig. A complete list of mass spectrometry identifications for the 1D BN-PAGE gel is provided in Figure S2 and Table S1 of the Supporting Information, and the corresponding identifications for 2D BN/SDS-PAGE are given in Table 1. The complete spectra and identification criteria have been deposited on-line as discussed in Materials and Methods.

Demonstration of Mitochondrial Kinase Activity by Radiolabeled 2D BN/SDS-PAGE. To screen for kinase-dependent phosphorylations within intact mitochondrial protein complexes, BN-PAGE gels were incubated with 350 μCi of [γ-^{32}P]ATP for 4.5 h at room temperature and subsequently resolved into individual subunits by SDS-PAGE. As shown in Figure 2C, significant ^{32}P labeling was observed for dozens of mitochondrial proteins. The overlay image of the [γ-^{32}P]ATP-labeled gel and the Coomassie blue-stained gel (Figure 2) reveals

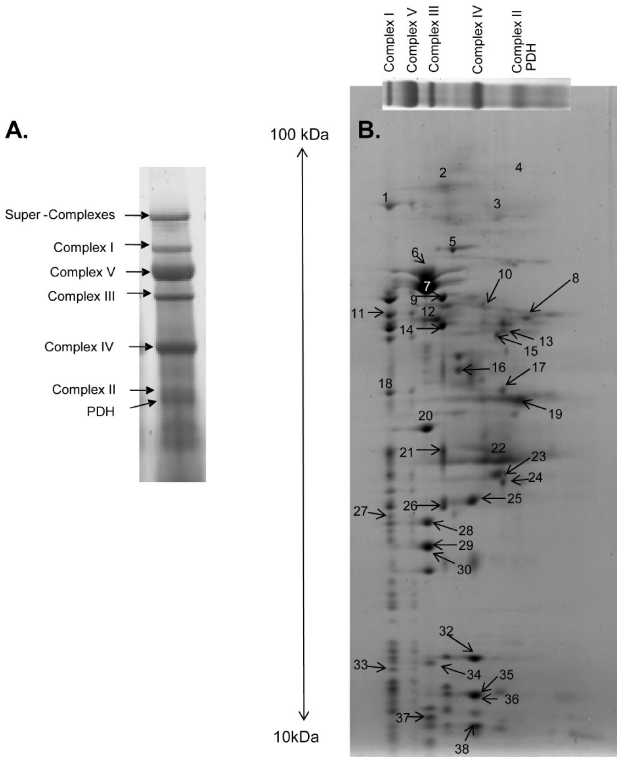


Figure 1. Mitochondrial protein identifications obtained by mass spectrometry. The mitochondrial complex identifications for a 1D BN-PAGE lane (A) and a 2D BN/SDS-PAGE gel (B). Numbers in panel B refer to the individual subunit identifications, which are listed in Table 1. Following 1D BN-PAGE, proteins are separated in the vertical direction by molecular mass, from ~150 to 10 kDa.

incorporation of ^{32}P into mitochondrial proteins involved with intermediary metabolism, energy transfer, and all five complexes of oxidative phosphorylation. Notably, intense ^{32}P labeling was observed for several PDH subunits, malate dehydrogenase, creatine kinase, adenine nucleotide translocase 1, and several subunits of Complexes IV and V. The presence of the PDH complex (PDHC) in these gels is surprising because the molecular mass of PDHC at 10 MDa far exceeds that of the normal mitochondrial oxidative phosphorylation complexes.[23] Thus, the consistent location of PDH subunits in this region of the gel likely represents reproducible partial breakdown of the PDHC. There were also a few low-concentration proteins that had very strong ^{32}P incorporation, but the levels were below the detection limits of mass spectrometry (i.e., unidentified proteins U1−U3 in Figure 2B). Importantly, these proteins do not likely represent subunits of the PDH or oxidative phosphorylation complexes, because their lack of Coomassie blue signal implies that they are not stoichiometric with the other subunits in the complexes. Additionally, a few proteins that we previously showed to be labeled with ^{32}P (i.e., branched chain keto-acid dehydrogenase and the E2 and E3 binding protein subunits of PDH[3]) were not detected in the current experiment, possibly because they were masked by other abundant proteins or not part of a larger protein complex and ran off the 1D BN-PAGE gel.

The reproducibility of this technique is demonstrated in Figure 3, where four [γ-^{32}P]ATP-labeled 2D BN/SDS-PAGE gels, from four different animals, are presented. Representative examples for the α and β subunits of Complex V, creatine kinase, Complex IV subunits IV, Va, Vb, and VIb, ANT1, and PDHE1α

Table 1. Mitochondrial Protein Identifications Obtained from a 2D BN/SDS-PAGE Gel by Mass Spectrometry[a]

2D BN-PAGE spot	protein annotation	protein accession number	no. of peptides
1	NADH dehydrogenase (ubiquinone) Fe−S protein 1, 75 kDa	Q66HF1	5
2	trifunctional enzyme, α subunit	P40939	2
3	Complex II, flavoprotein subunit	Q8HXW3	4
4	aconitase	P16276	3
5	heat shock protein 60, 60 kDa	P1 8687	2
6	Complex V, α subunit	P25705	11
7	Complex V, β subunit	P06576	7
8	citrate synthase	O75390	3
9	ubiquinol-cytochrome *c* reductase core protein I	P31800	2
10	fumarase	P10173	3
11	NADH dehydrogenase (ubiquinone) Fe−S protein 2, 49 kDa	P17694	7
12	creatine kinase	Q3ZBP1	3
13	pyruvate dehydrogenase, E1 α subunit	P08559	4
14	ubiquinol-cytochrome *c* reductase core protein II	P23004	2
15	long chain specific acyl-coenzyme A dehydrogenase	P51174	2
16	isocitrate dehydrogenase	Q28480	3
17	pyruvate dehydrogenase, E1 β subunit	P1 1177	2
18	NADH dehydrogenase (ubiquinone) 1α subcomplex, 9, 39 kDa	Q0MQB3	2
19	malate dehydrogenase	P00346	6
20	Complex V, γ subunit	P05631	5
21	ubiquinol-cytochrome *c* reductase, heme protein	Q9D0M3	2
22	ADP/ATP translocase 1	P48962	2
23	succinate dehydrogenase complex, subunit B	P21912	5
24	electron transfer flavoprotein, β subunit	Q6UAQ8	2
25	cytochrome *c* oxidase subunit II	P50667	3
26	ubiquinol-cytochrome *c* reductase, iron−sulfur subunit	P20788	4
27	NADH dehydrogenase (ubiquinone) flavoprotein 2, 24 kDa	P04394	4
28	Complex V, subunit b	P24539	3
29	Complex V, subunit O	P13621	2
30	Complex V, subunit d	AAI04565	2
31	peroxiredoxin-3	P35705	2
32	cytochrome *c* oxidase subunit IV	Q95283	3
33	NADH dehydrogenase (ubiquinone) 1β subcomplex, 4, 15 kDa	Q9CQC7	2
34	Complex V, δ subunit	P05630	2
35	cytochrome *c* oxidase subunit Va	P12787	3
36	cytochrome *c* oxidase subunit Vb	Q5S3G4	2
37	Complex V, subunit f	Q95339	2
38	cytochrome *c* oxidase subunit VIb	P00429	3

[a] These 38 proteins correspond to those labeled in Figure 1 and are referenced in Figures 2 and 5.

show that ^{32}P labeling is consistent between experiments. The extent of ^{32}P labeling observed with this strategy implies that active kinases are present in the mitochondrial complexes following resolution by BN-PAGE.

To further characterize ATP-dependent ^{32}P labeling, we conducted competition and chase experiments with cold ATP. In these experiments, BN-PAGE gels containing porcine heart mitochondria were incubated with [γ-^{32}P]ATP for 4.5 h, followed by a 4.5 h incubation with 1 mM cold (nonradioactive) ATP or incubated in cold ATP for 4.5 h, followed by a 4.5 h incubation with [γ-^{32}P]ATP. The results of these studies are shown in Figure 4. In a very similar fashion as observed for additions of inorganic ^{32}P to intact mitochondria,[24] chasing the hot ATP with cold ATP had little effect on the ^{32}P labeling of the proteins. In contrast, adding excess cold ATP before the [γ-^{32}P]ATP resulted in a significant and widespread decrease in the level of ^{32}P labeling. This behavior is consistent with an initial pool of ^{32}P association sites that once established do not turnover and the fact that if cold ATP occupies these sites first, the ^{32}P from ATP cannot displace it. These data suggest that the kinase activity, or on rate, far exceeds the phosphatase activity, or off rate, under these experimental conditions. An identical situation was reported for the addition of inorganic ^{32}P to isolated mitochondria being energized for the first time in vitro,[24] likely analogous to the addition of ATP to a blue native gel.

Attempted Verification of Mitochondrial Kinase Identification and Localization. Although the extent of ^{32}P labeling observed for mitochondrial proteins incubated with [γ-^{32}P]ATP provides strong evidence of kinase-mediated protein phosphorylation, the mitochondrial kinases resulting in the observed ^{32}P

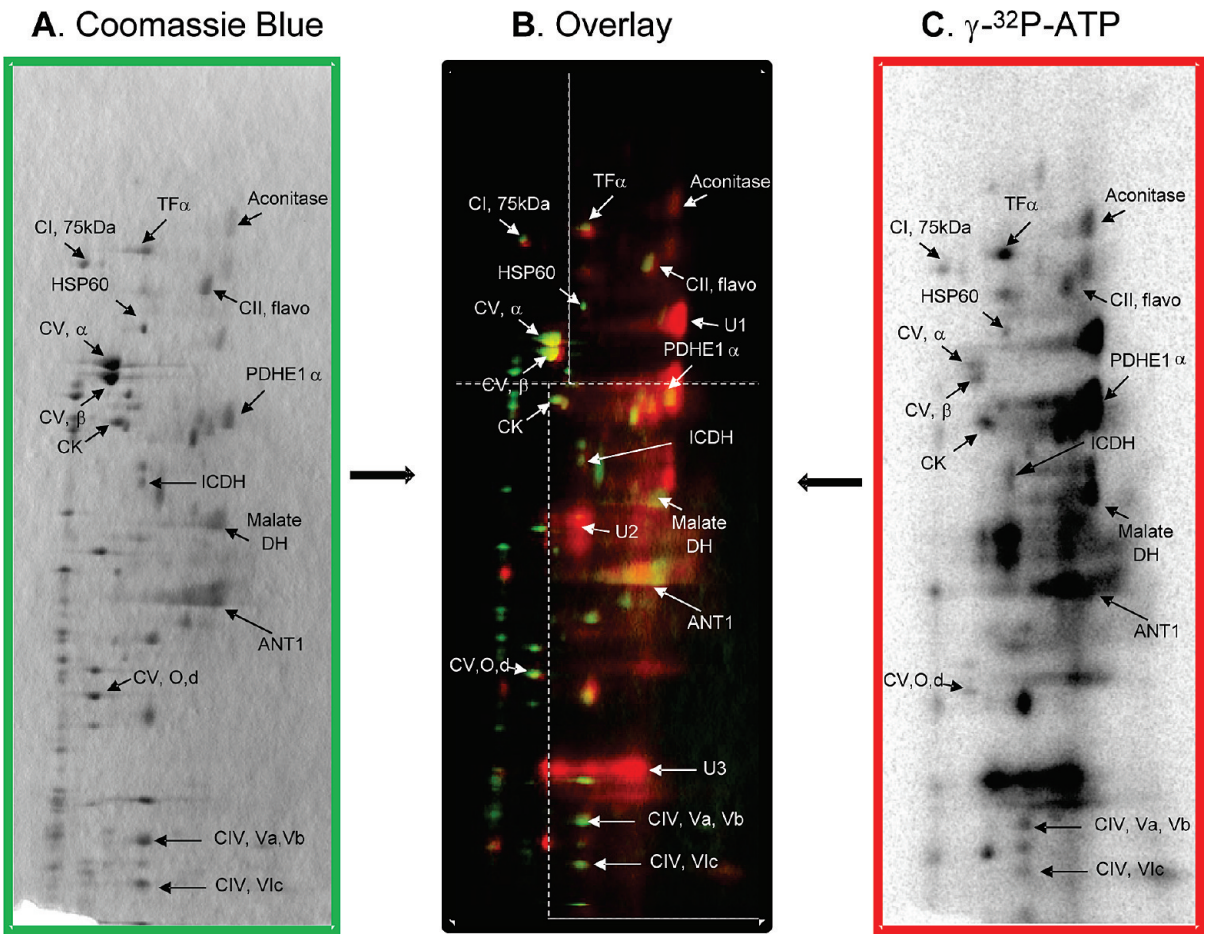


Figure 2. Overlay of a 2D BN/SDS-PAGE gel following incubation with [γ-^{32}P]ATP. The Coomassie blue-stained gel (A) is colored green in the overlay (B), and the [γ-^{32}P]ATP-labeled gel (C) is colored red. The overlay image in panel B applies different contrast levels across four regions of the gel image, as indicated by the white dotted lines. Following 1D BN-PAGE, proteins are separated in the vertical direction by molecular mass, from ~150 to 10 kDa. Figure abbreviations correspond to Table 1 as follows: CI, 75 kDa, protein 1; TFα, protein 2; CII, flavo, protein 3; aconitase, protein 4; HSP60, protein 5; CV, α, protein 6; CV, β, protein 7; CK, protein 12; PDHE1α, protein 13; ICDH, protein 16; malate DH, protein 19; ANT1, protein 22; CV, O, protein 29; CIV, Va, protein 35; CIV, Vb, protein 36; and CIV, VIc, protein 38. Additionally, proteins marked U1−U3 represent unidentified proteins 1−3, respectively.

labeling must be identified to reach such a conclusion. Thus, we coupled 2D BN/SDS-PAGE with antibody screening to identify specific kinases. PDK1 is a known mitochondrial kinase that phosphorylates PDHE1α. Figure 5A reveals strong ^{32}P labeling for the PDHE1α subunit, which implies that a functional PDK1 exists within the PDH complex. To confirm, a 2D BN/SDS-PAGE gel was probed for PDK1 in a paired experiment. As shown in Figure 5B, a specific protein localized to the PDH complex and matching the molecular mass of PDK1 (~48 kDa) is detected. Subsequent probing for PDK2, -3, and -4 with commercial antibodies was attempted but was not found to be as specific as that of the PDK1 antibody (results not shown). Targeted mass spectrometry revealed the presence of PDH phosphatase 1 and confirmed the identification of PDK2 (Figure 5C), with false discovery rates of less than 1%. This screen also confirmed the presence of PDK1 and PDK3, although the false discovery rates exceeded our 1% threshold. Collectively, these PDH complex results demonstrate the feasibility of our 2D BN/SDS-PAGE approach in screening for active mitochondrial kinases and phosphatases, though no kinases other than those previously assigned to the mitochondrial matrix were detected.

Screen for Additional Mitochondrial Kinases. Several studies in the literature report a role for cytosolic kinases that enter the mitochondria (despite no evidence of a mitochondrial leader sequence) and phosphorylate matrix proteins. Thus, using the same approaches that detected the PDH kinase and phosphatase system, 2D BN/SDS-PAGE gels were probed for a variety of kinases using Western blot analysis (Figure 5 and Figure S3 of the Supporting Information) and targeted mass spectrometry (Figure 5C). No evidence in the Western screens or in the targeted mass spectroscopy of classical cytosolic kinases was found in the pig mitochondrial oxidative phosphorylation complexes.

We also repeated the mass spectrometry studies in the blue native gels containing rat heart mitochondria where the protein database is better characterized. The BN-PAGE gels containing rat heart mitochondria also demonstrated incorporation of ^{32}P in a pattern similar to that of the pig in 2D BN/SDS-PAGE gels (Figure S5 of the Supporting Information). Protein identifications were conducted in the 2D BN/SDS-PAGE gel of the rat mitochondrial proteins (Figure S6 and Table S2 of the Supporting Information) as well as the intact 1D BN-PAGE bands (Figure S7 and Tables S3−S12 of the Supporting Information).

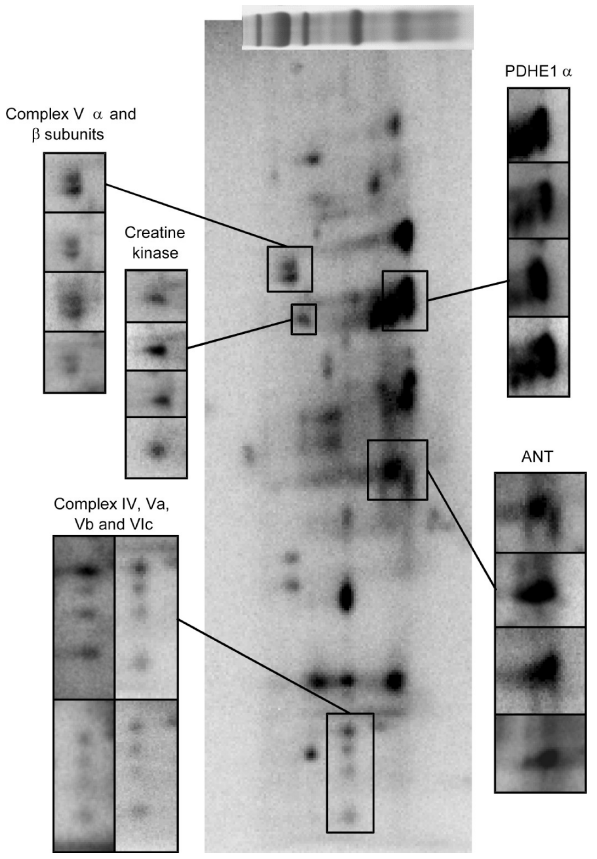


Figure 3. Reproducibility of a [γ-^{32}P]ATP-labeled 2D BN/SDS-PAGE gel. Individual panels show replicates from four different animals in different regions of the gel to demonstrate reproducibility. Each panel is labeled for the dominant ^{32}P-labeled protein in the magnified area. Following 1D BN-PAGE, proteins are separated in the vertical direction by molecular mass, from ~150 to 10 kDa.

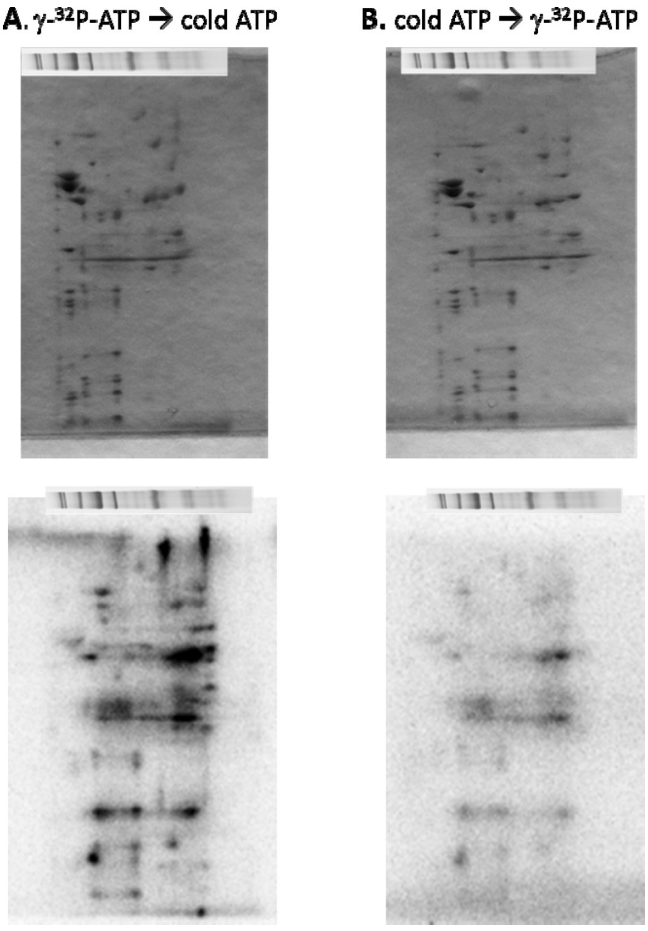
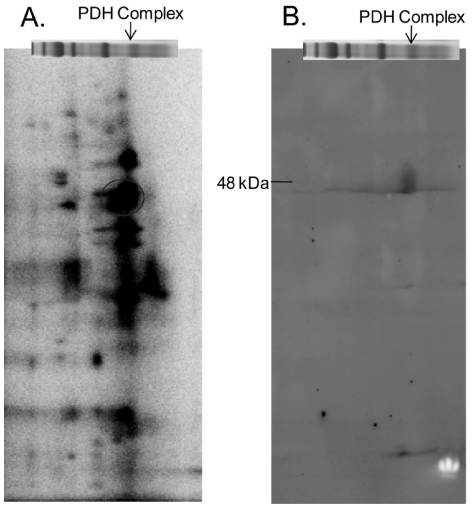


Figure 4. 2D BN/SDS-PAGE chase labeling experiment. The top panel shows the Coomassie blue-stained gel and the bottom panel the phosphor image. Panel A shows a 2D BN/SDS-PAGE gel labeled with [γ-^{32}P]ATP for 4.5 h, followed by a 4.5 h incubation with 1 mM cold (nonradioactive) ATP. Panel B shows the reverse, a 2D BN/SDS-PAGE gel labeled with cold ATP for 4.5 h, followed by a 4.5 h incubation with [γ-^{32}P]ATP.

Protein identifications were made with trypsin as well as chymotrypsin digestion, with many more peptides being detected with trypsin digestion. As found in the porcine data, no protein kinase reached significance within a mitochondrial complex in the rat. Even though the mitochondrial enzyme complexes demonstrate kinase activity in the rat and pig, these multiple screens did not reveal specific associations of cytosolic kinases with mitochondrial complexes. However, the PDH kinases were detected. This result led us to hypothesize that the kinase activity observed in this study may be due to ^{32}P metabolite associations, mimicking protein phosphorylations, or previously unknown autophosphorylation mechanisms within the complexes.

Demonstration of ^{32}P−Metabolite Association. We have previously shown that in crude mitochondrial extracts free inorganic phosphate can bind to succinyl CoA synthetase, α subunit (SCSα) and remain associated throughout the 2D electrophoresis process.[25] To test for the contribution of metabolite associations in these studies, we incubated BN-PAGE lanes with 350 μCi of [α-^{32}P]ATP, [γ-^{32}P]GTP, or ^{32}P for 4.5 h at room temperature. Incubation with [α-^{32}P]ATP can result in ATP, ADP, and/or AMP association, whereas incubation with [γ-^{32}P]ATP can result in kinase-mediated phosphorylation, autophosphorylation, and/or ATP association. Similarly, incubation with [γ-^{32}P]GTP can result in kinase-dependent phosphorylation, autophosphorylation, and/or GTP association. As shown in Figure 5B, several proteins were labeled with [α-^{32}P]ATP, including the α and β subunits of Complex V as well as heat shock protein 60 and CK (see the increased contrast image in Figure 5E). CK and several other proteins exhibited lower levels of incorporation of ^{32}P when labeled with [α-^{32}P]ATP relative to [γ-^{32}P]ATP, which implies that their incorporation of ^{32}P results mainly from protein phosphorylation, and not binding of ATP or ADP. In contrast, the extents of ^{32}P labeling for the α and β subunits of Complex V and heat shock protein 60 are approximately equal between [α-^{32}P]ATP and [γ-^{32}P]ATP incubations (Figure 5A,B), which suggests that these proteins are labeled as a result of ATP association and not protein phosphorylation. These findings are consistent with the apparent molecular mass shift associated with incorporation of ^{32}P for Complex V's β-subunit detected (Figure 6 and our earlier studies[3,26]) as well as reports of an ATP dependence for heat shock protein 60.[27] To screen for additional ^{32}P−metabolite associations, we conducted experiments in the presence of [γ-^{32}P]GTP (Figure 5C). As with [α-^{32}P]ATP and [γ-^{32}P]ATP, the α and β subunits of Complex V incorporated [γ-^{32}P]GTP. Whether this ^{32}P label results from phosphorylation or [γ-^{32}P]GTP association is unclear, as Complex V can



C.

	Complex I	Complex II	Complex III	Complex IV	Complex V	PDH Complex
KINASES						
Nucleoside diphosphate kinase B						•
Putative glycerol kinase 3	•					
Pyruvate dehydrogenase kinase, isozyme 1						○
Pyruvate dehydrogenase kinase, isozyme 2						•
Pyruvate dehydrogenase kinase, isozyme 3						○
PHOSPHATASES						
Pyruvate dehydrogenase phosphatase 1						•

Figure 5. Validation that 2D BN/SDS-PAGE can be used to screen for active mitochondrial kinases. PDH kinase 1 activity is demonstrated by the intense ^{32}P incorporation of the PDHE1α subunit (A), as highlighted by the circle. A representative Western blot following 2D BN/SDS-PAGE reveals a specific positive hit for PDH kinase 1, which is of the appropriate molecular mass (∼48 kDa) and localized to the region of the BN-PAGE gel that contains the PDH complex (B). Following 1D BN-PAGE, proteins are separated in the vertical direction by molecular mass, from ∼150 to 10 kDa. Panel C presents a list of kinases and phosphatases that were detected in Complexes I−V and the PDH complex using a combination of mass spectrometry and Western blot analysis. Filled circles denote proteins identified by targeted mass spectrometry, and empty circles denote proteins identified by Western blot analysis and mass spectrometry, but at a false discovery rate of >1%.

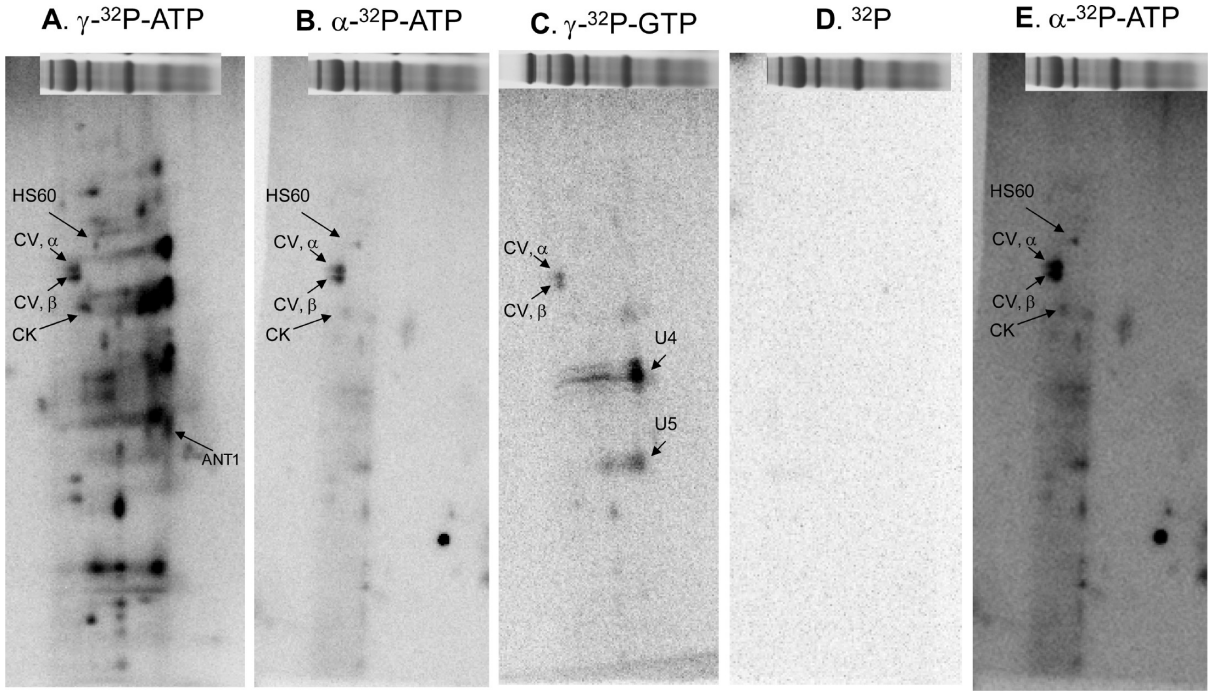


Figure 6. Comparison of 2D BN/SDS-PAGE ^{32}P labeling after 1D BN-PAGE incubation with different ^{32}P-labeled metabolites. 2D BN/SDS-PAGE gels following incubation of 1D BN-PAGE porcine heart gels with [γ-^{32}P]ATP (A), [α-^{32}P]ATP (B), [γ-^{32}P]GTP (C), ^{32}P (D), or [α-^{32}P]ATP with increased contrast (E). Abbreviations correspond to Table 1 as follows: HSP60, protein 5; CV, α, protein 6; CV, β, protein 7; CK, protein 12; and ANT1, protein 22. Additionally, proteins marked U4 and U5 represent unidentified proteins 4 and 5, respectively. Following 1D BN-PAGE, proteins are separated in the vertical direction by molecular mass, from ∼150 to 10 kDa.

hydrolyze both ATP and GTP with similar affinities (Figure S8 of the Supporting Information). A few additional proteins were labeled with [γ-^{32}P]GTP, notably two unidentified proteins (U4 and U5) that were labeled intensely with [γ-^{32}P]GTP. Finally, incubation of BN-PAGE lanes with ^{32}P alone did not result in detectable radiolabeling (Figure 5D). This is important because, as mentioned above, incubation of BN-PAGE lanes with [γ-^{32}P]ATP or -GTP generates free ^{32}P and ADP or GDP at the level of Complex V. The lack of free ^{32}P labeling confirms that the vast majority of ^{32}P incorporation observed in these studies results from ATP-dependent covalent processes, rather than artifacts from metabolite associations and the high sensitivity

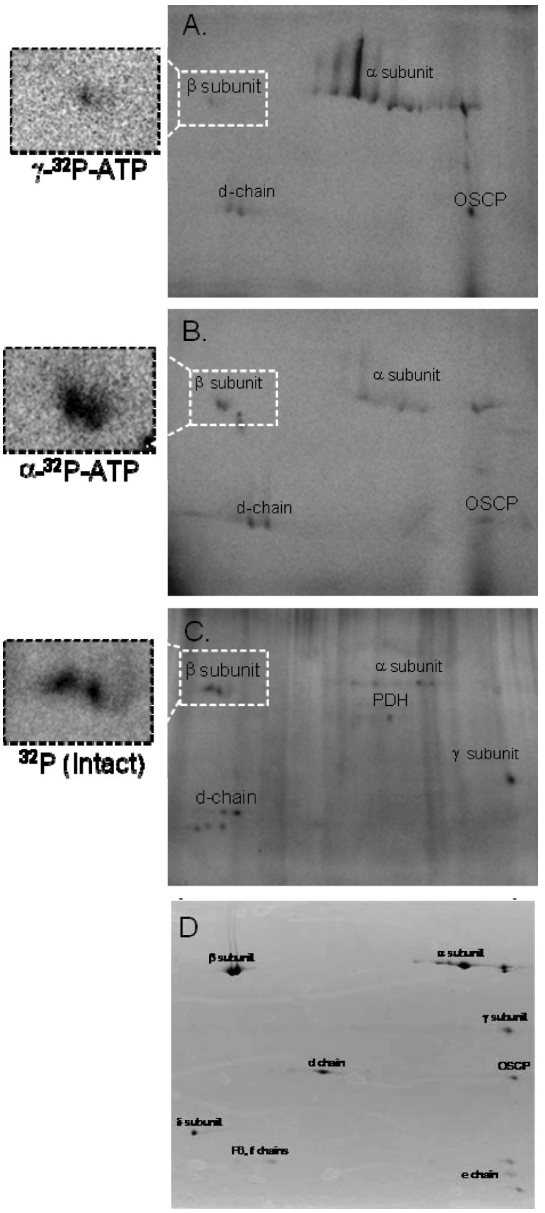


Figure 7. Purified Complex V radiolabeled in vitro with ATP and in intact mitochondria. Panels A and B show Complex V labeled in vitro with [γ-^{32}P]ATP and [α-^{32}P]ATP, respectively. Panel C shows the labeling pattern of Complex V purified from intact mitochondria following incubation with ^{32}P. Proteins are separated in the horizontal direction by isoelectric focusing point, from pH 3 to 10, and vertically by molecular mass, from ~150 to 10 kDa. Panel D shows a representative protein stain of immunocaptured porcine Complex V as previously described.[24] All assignments were made by using fiduciary markers on the ^{32}P- and protein-stained gel as previously described.[24]

of ^{32}P. These studies did not reveal the previously described binding of ^{32}P to SCSα,[25] because this protein was not retained as a complex in this preparation, according to mass spectrometry screens. Overall, these studies demonstrate that a majority of the ^{32}P labeling observed in these 2D BN/SDS-PAGE gels is dependent on [γ-^{32}P]ATP and likely the result of protein phosphorylation. The only tight metabolite incorporations seem to be associated with the α and β subunits of Complex V, heat shock protein 60, CK, and a few unidentified proteins.

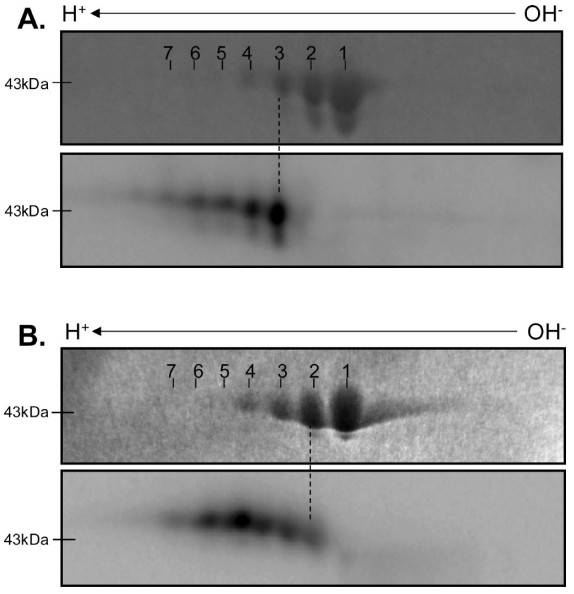


Figure 8. Purified creatine kinase radiolabeled in vitro with ATP. Panels A and B show incorporation of [γ-^{32}P]ATP and [α-^{32}P]ATP into creatine kinase, respectively. For both panels, the Coomassie blue-stained image is above the radiolabeled image. Proteins are separated in the horizontal direction by isoelectric focusing point, from pH 3 to 10, and vertically by molecular mass, from ~150 to 10 kDa.

In Vitro Demonstration of Mitochondrial Autocatalytic Processes. Because exogenous mitochondrial kinases have been difficult to identify, we hypothesized that ATP-dependent autophosphorylation events may be responsible for a majority of the protein kinase activity detected in this study. To evaluate this further, we compared the ^{32}P labeling patterns of purified Complex V incubated in vitro, with [γ-^{32}P]ATP (Figure 7A) or [α-^{32}P]ATP (Figure 7B), with that of Complex V isolated from intact mitochondria that were labeled with ^{32}P under energized conditions (Figure 7C). In vitro [^{32}P]ATP association was shown for the α, β, d, and OSCP subunits, whereas intact ^{32}P incorporation showed labeling for the α, β, γ, and d chain subunits, as we previously demonstrated.[3,26] For the [^{32}P]ATP studies, the α and OSCP subunits were much more intensely labeled with [γ-^{32}P]ATP than with [α-^{32}P]ATP, consistent with a rapid autophosphorylation for these subunits. With regard to Complex V's β subunit, incubation with [γ-^{32}P]ATP showed weak ^{32}P incorporation, whereas incubation with [α-^{32}P]ATP revealed strong labeling and increasing molecular mass for the ^{32}P-labeled component of the β subunit, similar to Figure 6B and our previous observations in intact mitochondria.[3,26] The [α-^{32}P]ATP labeling pattern for the β subunit, coupled with our findings in Figure 7B, suggests that labeling of the β subunit primarily results from ^{32}P−metabolite associations such as ADP and/or ATP binding. It is important to point out that unlike the β subunit, which has catalytic metabolite binding sites, the α, d, and OSCP subunits do not, and thus, it is unlikely that these interactions are related to metabolite binding sites. As described above, incubating purified Complex V with [γ-^{32}P]ATP results in free ^{32}P. To ensure that these autocatalytic studies were not influenced by Complex V's hydrolytic process, we also incubated purified Complex V with free ^{32}P but detected no labeling (results not shown). Comparing the in vitro ^{32}P labeling pattern of purified Complex V (Figure 7A,B) with the pattern obtained

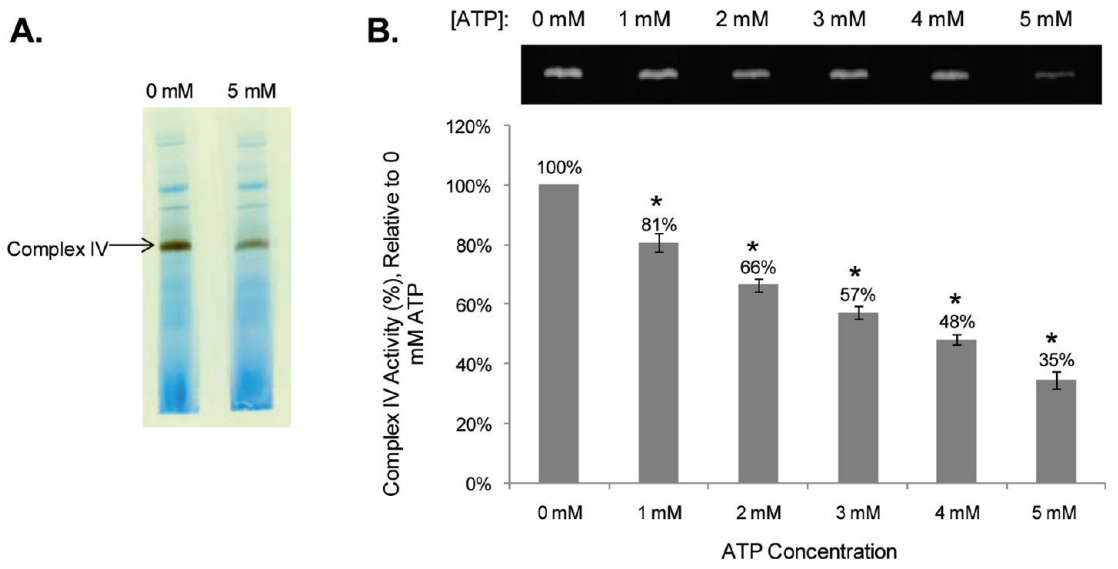


Figure 9. Inhibition of Complex IV activity by ATP. Panel A provides a representative example of the colorimetric Complex IV activity assay in the presence of 0 and 5 mM ATP. Complex IV activity over an ATP dose response, ranging from 0 to 5 mM ATP, is shown in panel B. The graph in panel B represents four different biological samples. Data are displayed as a percentage of Complex IV activity, relative to the 0 mM ATP condition.

with intact mitochondria (Figure 7C) revealed many similarities, with the notable exceptions of OSCP, which was only labeled in vitro with [^{32}P]ATP, and the γ subunit, which was labeled only in intact mitochondria. These data suggest that a majority of Complex V's ^{32}P labeling is due to autocatalytic processes that occur within the enzyme complex, whereas the γ subunit is likely phosphorylated by a matrix kinase that is not tightly associated with Complex V or the configuration of the complex in the gels inhibits autophosphorylation of this subunit.

To demonstrate that autocatalytic processes were not unique to Complex V, we also screened purified creatine kinase (CK). CK was selected for several reasons. (1) It plays an important role in intracellular energy transfer. (2) It is labeled strongly with [γ-^{32}P]ATP and weakly with [α-^{32}P]ATP in our 2D BN/SDS-PAGE studies (Figure 6). (3) There is a debate in the literature about whether CK is autophosphorylated[20,28,29] or nucleotidylated (i.e., autoincorporation of the entire adenosine molecule).[30] For these studies, purified CK from the cytosolic MM fraction was used instead of the mitochondrial fraction because it was commercially available at very high purity. Incubating CK in vitro with [γ-^{32}P]ATP (Figure 8A) revealed that the most intense ^{32}P incorporation occurred in the third isoelectric variant, whereas [α-^{32}P]ATP labeling (Figure 8B) was most intense in the fifth isoelectric variant. The differential ^{32}P labeling of CK's isoelectric variants shows that a greater mole fraction of the protein is labeled with [γ-^{32}P]ATP than with [α-^{32}P]ATP, consistent with the data presented in panels A and B of Figure 7. It should be stressed that extreme care was taken in aligning these gels using four fiduciary points on each gel. This finding suggests that two mechanisms are contributing to CK's ^{32}P labeling; we propose that autophosphorylation is dominating the [γ-^{32}P]ATP labeling, while the weaker [α-^{32}P]ATP labeling reflects nucleotidylation of either AMP or ADP. Thus, both mechanisms debated in the literature seem to be in play for this enzyme, albeit to different extents.

Functional Effects of ATP on Complex IV Activity in BN-PAGE Gels. To determine if direct incubation of the BN-PAGE lanes with ATP altered enzyme activity, we monitored Complex IV activity in the presence of various ATP concentrations. Complex IV was selected because previous studies have shown that it is inhibited by ATP.[31−33] Furthermore, because ATP is not involved in the catalytic process of Complex IV, as is the case for Complex V, any allosteric effect of ATP on this complex could be directly determined in the BN-PAGE gels. Toward this end, we incubated individual BN-PAGE lanes with ATP contents ranging from 0 to 5 mM. Complex IV activity was shown to decrease with increasing concentrations of ATP, relative to the 0 mM ATP condition (Figure 9). Whether this ATP-induced inhibition is due to phosphorylation by a kinase associated with CIV, autophosphorylation, or another allosteric mechanism has yet to be determined. Figure 10 reveals that Complex IV activity recovers in the absence of ATP or with protein phosphatase 1 (PP1), consistent with previous reports,[34] further demonstrating that the mitochondrial enzyme complexes are dynamically regulated by ATP.

DISCUSSION

This study provides further support for the notion that mitochondrial protein phosphorylation is extensive and ubiquitous within the enzymes associated with energy metabolism. Widespread ^{32}P labeling was directly observed in BN-PAGE gels in vitro, with the addition of [γ-^{32}P]ATP. ATP is not a metabolic substrate for Complexes I−IV; thus, incorporation of ^{32}P from [γ-^{32}P]ATP is not associated with the normal metabolic process. Thus, in these complexes, the extensive ^{32}P incorporation demonstrated that, aside from the well-recognized energetic functions, they have protein kinase activity. Surprisingly, with the exception of the PDH system, our attempt to detect protein kinases within isolated mitochondrial complexes, using targeted mass spectrometry and Western blot analysis, failed to detect any previously recognized protein kinase. This result suggests that conventional cytosolic kinases may not exist within the mitochondrial complexes but, rather, that these protein complexes have intrinsic kinase activity. Further evidence of autophosphorylation within the mitochondrial complexes was obtained by incubation of purified Complex V and CK with [α-^{32}P]ATP and

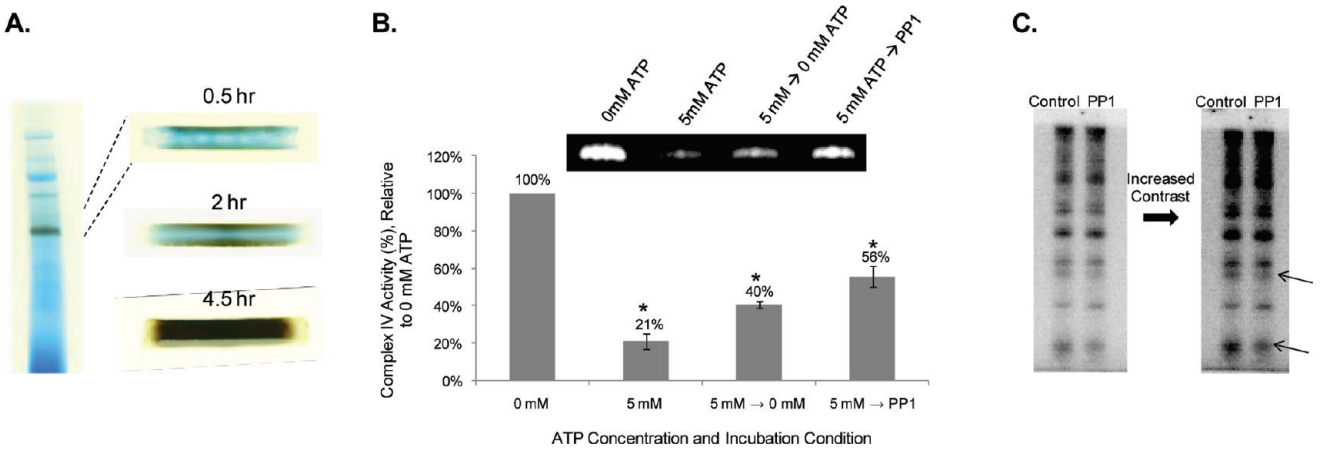


Figure 10. Diffusion limitation of in-gel activity assays. Panel A shows that several hours are required for the small molecules used in the colorimetric Complex IV activity assay to penetrate the 1 mm thick BN-PAGE gels. Complex IV activity measurements in response to the absence of ATP and presence of PP1 are shown in panel B. The graph in panel B represents four different biological samples, and data are displayed as a percentage of Complex IV activity, relative to the 0 mM ATP condition. Panel C shows low- and high-contrast images of the Complex IV subunits, following incubation with [γ-^{32}P]ATP for 4.5 h and a 4.5 h wash (Control) or a 4.5 h incubation in PP1 (PP1). The arrows in panel C point to two potential sites of regulation.

[γ-^{32}P]ATP in vitro and comparison of their labeling pattern to those of proteins purified following incorporation of ^{32}P into intact mitochondria. With the exception of Complex V's γ subunit, the ^{32}P labeling patterns for Complex V in the in vitro and intact mitochondria conditions were very similar. Finally, this study revealed that the addition of ATP to the BN-PAGE gels decreased Complex IV activity that was partially reversed by phosphatase, consistent with an inhibitory protein phosphorylation in this complex.[35]

Detection of Protein Kinases in the Mitochondrial Complexes. The potentially low concentration of mitochondrial protein kinases in total protein mixtures and the uncertainty of isolation artifacts make the detection of matrix protein kinases a major challenge. Take, for instance, PDH kinase 1 (PDK1). Previous studies have shown that only one or two copies of PDK dimers exist per PDH complex.[36] This low mole fraction, among complex mitochondrial protein mixtures, generally places PDK1 below the detection level for most gross mass spectrometry or gel electrophoresis staining efforts. To this point, the most comprehensive mitochondrial proteome study to date by Pagliarini et al.[15] did not detect the PDH kinases or virtually any other mitochondrial protein kinases. The 2D BN/SDS-PAGE strategy described here was specifically designed to circumvent these difficulties, by directly assaying for mitochondrial protein kinase activity and screening for protein kinase identifications within the individual complexes. Thus, this approach would enhance the mole fraction of the kinase molecules in complex mitochondrial protein mixtures. This strategy was validated via detection of several low-abundance protein kinases and phosphatases within the well-studied PDH complex. However, even with this enhanced sensitivity, we detected no additional mitochondrion-localized protein kinases, including those that have been previously shown to associate with intact mitochondria.

The fact that each complex revealed specific protein kinase activity in the gels suggests that the kinase activity is intrinsic to the complex's proteins. That is, the protein kinase should be roughly equal in mole fraction to the proteins within the complex. Because we detected a majority of the subunits for the oxidative phosphorylation complexes, with the exception of the very low molecular mass elements or very hydrophobic elements, it is unlikely that the concentration is limiting. As further support for this notion, the PDH complex exists at a much lower concentration (>100-fold less) than the oxidative phosphorylation complexes (see Table 2, calculated according to refs 37 and 38). The 2D BN/SDS-PAGE approach detected PDH kinases 1, 2, and 3 as well as PDH phosphatases. Thus, the concentration of the oxidative phosphorylation complexes of protein kinases and/or phosphatases should far exceed the sensitivity limit of this approach if the stoichiometry of kinases and/or phosphatases to target proteins is similar in the PDH and oxidative phosphorylation protein complexes. It is possible that because of the extreme sensitivity of ^{32}P, this assay is able to detect protein kinase activity that affects only a small fraction of the total protein. In other words, protein phosphorylation may be exhibited by only a small percentage of each protein subunit. However, numerous mitochondrial protein phosphorylations have been detected using less sensitive techniques, such as gross mass spectrometry, isoelectric shifts, and phosphorylation sensitive dyes,[5,6,8,39,40] implying that a substantial fraction of the proteins is phosphorylated. Additionally, the considerable inhibition of Complex IV by ATP implies that phosphorylation is able to modulate a large percentage of Complex IV's protein subunit(s) in the gel environment. However, the mole fraction of proteins that are phosphorylated under the conditions used in this study and the specific amino acid sites of the ^{32}P association have yet to be determined.

What Is the Nature of Association of ^{32}P with the Mitochondrial Protein Complexes?. The absence of classical protein kinases among the mitochondrial complexes suggests that the protein phosphorylation processes might be very different from the kinase systems well characterized in the mammalian cytosol. An obvious question becomes what mechanism(s) are responsible for the widespread association of ^{32}P with mitochondrial proteins, as observed in this study and many others.[3−10] One suggestion has been that the protein phosphorylations detected by mass spectrometry screen or dyes are the result of phosphorylations that occurred in the cytosol before the protein was transported into the matrix. Though this might be the case for

Table 2. Numbers of Protein Complex Molecules per Mitochondrion, for PDH Complex and Complexes I−V of Oxidative Phosphorylation[a]

	no. of complex molecules/cell	no. of complex molecules/mitochondrion
PDH Complex	93372	19
Complex 1	6287030	1257
Complex II	9430545	1886
Complex III	18861089	3772
Complex IV	40865693	8173
Complex V	22004604	4401

[a] These values were calculated from refs 37 and 38, and 5000 mitochondria per heart cell were assumed.

some matrix protein phosphorylations, the fact that we are detecting ^{32}P incorporations in isolated mitochondria and isolated complexes demonstrates that this pool of protein phosphorylations we are evaluating is generated within the mitochondria, consistent with protein kinase activity in the matrix. It is very difficult to correlate specific phosphorylation sites detected with protein mass spectrometry with ^{32}P association. Experimental approaches might include simultaneous studies on isolated peptides using ^{32}P detection and mass spectroscopy or amino acid substitution effects on ^{32}P incorporation.

Is the ^{32}P labeling protein phosphorylation or tight metabolite association? We recently demonstrated that noncovalent binding of P_i to SCSα survives the denaturing conditions of SDS gel electrophoresis.[25] Thus, to resolve the contribution of protein phosphorylation to the ^{32}P-labeled proteins observed in this study, we performed several controls, including incubation with free ^{32}P to control for inorganic phosphate (P_i) binding, [α-^{32}P]ATP to control for ADP and/or ATP association, and [γ-^{32}P]GTP to control for GTP metabolites. No P_i binding was detected in the 2D-BN-PAGE study (Figure 6) or in the purified Complex V studies (Figure 8) upon incubation with ^{32}P. However, strong [α-^{32}P]ATP labeling was observed for the α and β subunits of Complex V and heat shock protein 60, along with weak association with other mitochondrial proteins, including CK. We confirmed the existence of these reactions by labeling purified CK with [α-^{32}P]ATP and [γ-^{32}P]ATP (Figure 9). Additionally, a few proteins were labeled with [γ-^{32}P]GTP, including the α and β subunits of Complex V. Therefore, these experiments show that a relatively small degree of metabolite association contributes to the overall ^{32}P labeling pattern observed in this study. Furthermore, a majority of the ^{32}P-labeled proteins were labeled only in the presence of [γ-^{32}P]ATP, implying that they underwent ATP-dependent protein phosphorylation. A striking example of this involved ANT1 (Figure 7). ANT1 functions as a gated pore through which ADP and ATP are exchanged between the mitochondrial matrix and the cytoplasm. Thus, while ANT1 contains ADP and ATP binding sites, it was not labeled with [α-^{32}P]ATP but was labeled with [γ-^{32}P]ATP, providing strong evidence of protein phosphorylation and not metabolite binding in this important transporter. The possible role of metabolite associations is further discussed in the section on limitations.

Because mitochondria originated from bacteria and the PDH and branch chain dehydrogenase kinase system shares more sequence homology with bacterial histidine kinases than eukaryotic cytosolic kinases,[41] it was logical to search for bacterial characteristics, rather than the conventional eukaryotic kinase targets and pathways within the mitochondrial protein phosphorylations observed here. Bacterial proteins are phosphorylated using a two-component system, which generally involves histidine autophosphorylation and subsequent phosphotransfer to aspartate residues.[42] It is important to point out that several conventional serine, threonine, and tyrosine protein phosphorylations have now been found in bacteria (for a review, see ref 43); thus, protein phosphorylations are not limited to two-component systems in bacteria. Although eukaryotic proteins have also been shown to autophosphorylate[44] to a variety of sites, recently regulatory phosphohistidines have also been described in the eukaryotic cell, such as the mitochondrial branch chain kinase[45] as well as G-proteins and membrane channels.[46] Interestingly, one of the only other protein kinases detected in this study, nucleoside diphosphate kinase (NDPK) (Figure 5C), undergoes autophosphorylation on its active histidine residue.[47,48] Furthermore, studies have suggested that this phosphate is transferred from the unstable phosphohistidine to a nearby serine residue,[48] while others suggest that direct serine autophosphorylation can occur, depending on the concentration of metabolites in the cellular environment.[49]

The extensive widespread autophosphorylation detected in this study, the bacterial origins of the mitochondria, and the homology of the phosphohistidine kinases to the protein kinases undisputedly in the matrix along with NDPK make the possibility that some of these sites could be phosphohistidine or phosphoaspartate or other bacterial protein phosphorylation sites a reasonable consideration. However, the detection of phosphohistidine or phosphoaspartate is extremely difficult because of the lability of these bonds,[46] especially to the acid conditions normally used in proteomic procedures and the lack of specific antibodies. In early studies of association of ^{32}P with mitochondrial proteins, it was found that the majority (>90%) of the ^{32}P associations were acid labile.[50] Boyer et al. described one of these sites as an enzyme catalysis phosphohistidine in succinate thiokinase.[51,52] Consistent with this notion that many mitochondrial phosphorylation sites are acid labile were our own observations in BN-PAGE or Ghost native gel electrophoresis gels from ^{32}P-labeled mitochondria[24] that demonstrated the vast majority of the ^{32}P association was acid labile in these minimally solubilized systems consistent with the bacterial protein phosphorylation system (i.e., phosphohistidine, phosphoaspartate, etc.) along with possible metabolite associations. Thus, it is possible that some of the ^{32}P protein associations detected in this study could be small remaining fractions of a phosphohisidine or phosphoaspartate that survived the electrophoresis process. To test this hypothesis further will require the development of significantly different protein isolation techniques and protein phosphorylation detection schemes.[46]

Collectively, these findings suggest that mitochondrial protein phosphorylation may be governed by widespread complex autophosphorylation and/or phosphotransfer mechanisms. As

this implies that mitochondrial protein phosphorylation may be more similar to the bacterial two-component system than traditional diffusing eukaryotic cytosolic kinases, it is apparent that the extent of protein phosphorylation decreases significantly after the isolation of mitochondria or creation of the BN-PAGE gels based on the observation that initial and nearly irreversible phosphoprotein pools build up upon reintroduction of ATP into the mitochondrial matrix[24] or within the BN-PAGE gels (Figure 4). This is also consistent with the original studies of PDHK by Randle[53] that also showed a dephosphorylation after the isolation of mitochondria that was rapidly reversed with re-energization and warming. The simplest mechanism for explaining this behavior is that the ratio of kinase to phosphatase activity is increased with ATP concentration. At high ATP concentrations, the kinase activity far exceeds the phosphatase activity, explaining the rapid net phosphorylation of protein during rewarming or reintroduction of ATP as well as the fact that once phosphorylated the turnover is extremely slow, revealed in the cold ATP chase experiments, suggesting a very low phosphatase activity at the steady state. These data also suggest that great care must be taken in assuming that freshly isolated mitochondria, for functional studies, reflect the post-translational modification state of the mitochondria, in vivo.

Study Limitations: Metabolite Associations, Diffusion Limitations, and Mole Fractions of Phosphorylated Protein. As mentioned above, it is important to recognize that metabolite associations involving P_i binding,[25] nucleotidylation,[30] and ADP ribosylation[54] coupled to NAD^+ synthesis via NMNAT-3[55] have all been shown to occur in the mitochondrial matrix. It is therefore necessary to take great care when assigning ^{32}P associations as phosphorylation events. We focused further attention on this topic by comparing the in vitro ^{32}P labeling patterns of purified Complex V (Figure 7) with the ^{32}P labeling profile obtained from intact mitochondrial studies.[3,5,26] Unlike the α, β, and d subunits, which were labeled in vitro with [α-^{32}P]ATP and [γ-^{32}P]ATP (Figures 6 and 7) and with ^{32}P in intact mitochondria,[3] the γ subunit is labeled only in intact mitochondria. This finding implies that ^{32}P labeling of the γ subunit is not "autocatalytic" within Complex V in the gel environment. As the 2D BN/SDS-PAGE studies described here did not reveal ^{32}P labeling for Complex V's γ subunit, it provides further evidence that γ subunit phosphorylation is dependent on a mitochondrial kinase that is not tightly bundled with Complex V, cofactors for the reaction are missing, or the conformation of Complex V in the native gel inhibits this process.

Another important consideration for this study is the diffusion limitation of assaying directly in BN-PAGE gels. As shown in Figure 10A, several hours are required for small molecules, such as ATP, GTP, P_i, and Ca^{2+}, to penetrate the 1 mm thick gels. Because this study demonstrated that Complex IV activity was inhibited by ATP (Figure 8) and recovered in the absence of ATP and with PP1 (Figure 10B), we next attempted to correlate these changes in activity with changes in phosphorylation (Figure 10C). Unfortunately, because of the large size of PP1 (~38 kDa), this phosphatase was unable to penetrate the BN-PAGE gel and act on the ^{32}P-labeled Complex IV subunits in their entirety. Instead, PP1 accessed only the surface of the BN-PAGE gel, an estimated 15% of Complex IV. This explains the discrepancy between our activity measurement and the ^{32}P data, that is, why a large change in Complex IV activity was observed, with very small changes in the ^{32}P labeling profile. Because the Complex IV activity assay is run for 30 min, only the protein molecules on the surface are being assayed. Hence, these surface molecules are the same Complex IV molecules upon which PP1 acts, and the activity assay reflects the presumably large mole fraction of Complex IV that is modified by PP1. However, in the ^{32}P labeling study, ~100% of the Complex IV molecules are exposed to ^{32}P, but only ~15% of them are exposed to PP1. This finding points out that the diffusion limitation of large protein molecules, such as phosphatases, must be considered when conducting this type of analysis in the gel environment. It is also important to point out that these in-gel BN-PAGE assays are evaluating only partial reactions of the complexes outside of the normal membrane environment as well as without a membrane potential. Thus, these assays can be used only as extrapolations to what might be happening in the inner mitochondrial membrane.

Finally, using ^{32}P labeling, it is difficult to extrapolate these data to the total mole fraction of an enzyme or complex that is phosphorylated. For signaling molecules, small fractions of a given protein phosphorylation can result in an amplified signal. However, the majority of the phosphorylation sites detected in this study were metabolic enzymes in which the only way to significantly influence the activity is to impact a significant fraction of the enzymes.[38] While ^{32}P provides insight into protein phosphorylation turnover, it is not very useful in determining the mole fraction of a given protein that is phosphorylated. In addition, the dynamic nature of protein phosphorylation could also complicate this interpretation. ^{32}P labeling, though very sensitive, is a poor marker of the fraction of a protein phosphorylated at any given time. The degree of ^{32}P protein labeling is dependent on turnover of the sites, as well as the total amount of protein phosphorylated. Thus, in the two extremes, a protein could be highly phosphorylated and not turn over, providing no labeling at all, while a partially phosphorylated protein with a high rate of turnover could generate a large ^{32}P labeling signal. These complications are why we are interpreting our results as the detection of protein kinase activity, not the degree of phosphorylation of the mitochondrial protein complexes. Further studies are required to establish the mole fraction and specific amino acids of the labile protein phosphorylation sites detected in this study, as well as their impact on enzyme function and mechanistic details of the intrinsic protein kinase/phosphatase system.

SUMMARY

This study demonstrates that the mitochondrial complexes of oxidative phosphorylation have intrinsic protein kinase activity, with virtually no evidence of conventional cytosolic kinases within these complexes. With the exception of the γ subunit of Complex V, which may require a matrix-dependent kinase reaction, most of the ^{32}P incorporations detected in intact mitochondria were detected in BN-PAGE-resolved complexes exposed to [γ-^{32}P]ATP. This protein kinase activity may represent unrecognized protein kinase motifs in these protein complexes of bacterial origin or highly efficient coupling of a few copies of kinases not detected in this system. It was also demonstrated that the addition of ATP to isolated Complex IV inhibited its enzyme activity, consistent with an inhibitory action of autophosphorylation reactions or allosteric effects. Further work is required to determine the nature and position of these phosphorylation sites, especially in Complexes I, III, and IV, which are not classically believed to interact or require ATP for their electron transport function, as well as the kinase and phosphatase system regulating this process.

■ ASSOCIATED CONTENT

Supporting Information. Linearity of the Complex IV in-gel activity assay (Figure S1), 1D BN-PAGE of porcine heart mitochondria (Figure S2), mass spectrometry identification of proteins in the complex bands of Figure S2 (Table S1), demonstration that Abgent's pyruvate dehydrogenase kinase 4 antibody primarily detects the α subunit of Complex V, not PDK4 (Figure S3), negative 2D Western blots of MEK6, PKCδ, and PKCε (Figure S4), association of rat mitochondria with ^{32}P in 1D BN/2D SDS-PAGE gels after incubation with [γ-^{32}P]ATP in the 1D BN-PAGE gel (Figure S5), 1D BN/2D SDS-PAGE of rat heart mitochondrial proteins (Figure S6), protein identifications in a rat 1D BN/2D SDS-PAGE gel (Table S2), 1D BN-PAGE gel of rat heart mitochondrial proteins (Figure S7), Complex I band chymotrypsin digestion (Table S3), Complex I band trypsin digestion (Table S4), Complex II band chymotrypsin digestion (Table S5), Complex II band trypsin digestion (Table S6), Complex III band chymotrypsin digestion (Table S7), Complex III band trypsin digestion (Table S8), Complex IV band chymotrypsin digestion (Table S9), Complex IV band trypsin digestion (Table S10), Complex V band trypsin digestion (Table S11), Complex V band chymotrypsin digestion (Table S12), and hydrolysis of ATP and GTP by porcine Complex V (Figure S8). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

BN-PAGE, blue native polyacrylamide gel electrophoresis; 1D BN-PAGE, one-dimensional blue native polyacrylamide gel electrophoresis; 2D BN/SDS-PAGE, two-dimensional blue native polyacrylamide gel electrophoresis; PDH, pyruvate dehydrogenase; BCKDH, branched chain α-ketoacid dehydrogenase; PDK1, pyruvate dehydrogenase kinase 1; CK, creatine kinase; RCR, respiratory control ratio; ANT1, ADP/ATP translocase 1; OSCP, oligomycin sensitivity conferring protein; PP1, protein phosphatase 1.

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