

cAMP-dependent Tyrosine Phosphorylation of Subunit I Inhibits Cytochrome *c* Oxidase Activity*

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Signaling pathways targeting mitochondria are poorly understood. We here examine phosphorylation by the cAMP-dependent pathway of subunits of cytochrome *c* oxidase (COX), the terminal enzyme of the electron transport chain. Using anti-phospho antibodies, we show that cow liver COX subunit I is tyrosine-phosphorylated in the presence of theophylline, a phosphodiesterase inhibitor that creates high cAMP levels, but not in its absence. The site of phosphorylation, identified by mass spectrometry, is tyrosine 304 of COX catalytic subunit I. Subunit I phosphorylation leads to a decrease of V_{\max} and an increase of K_m for cytochrome *c* and shifts the reaction kinetics from hyperbolic to sigmoidal such that COX is fully or strongly inhibited up to 10 μ M cytochrome *c* substrate concentrations, even in the presence of allosteric activator ADP. To assess our findings with the isolated enzyme in a physiological context, we tested the starvation signal glucagon on human HepG2 cells and cow liver tissue. Glucagon leads to COX inactivation, an effect also observed after incubation with adenylyl cyclase activator forskolin. Thus, the glucagon receptor/G-protein/cAMP pathway regulates COX activity. At therapeutic concentrations used for asthma relief, theophylline causes lung COX inhibition and decreases cellular ATP levels, suggesting a mechanism for its clinical action.

Cytochrome *c* oxidase (COX),¹ the terminal enzyme of the mitochondrial respiratory chain, reduces oxygen to water and pumps protons across the inner mitochondrial membrane. COX contains 13 subunits per monomer, three of which are encoded by the mitochondrial genome. COX has been shown to be the rate-limiting enzyme of oxidative metabolism under physiological conditions in a variety of human cell types (1) and in a mouse cell line with a mutation in COX subunit I (2). The functional mammalian enzyme has been crystallized as a dimer (3) and shows three features, described below, that are

found in key metabolic enzymes: allosteric regulation, isoforms, and phosphorylation. COX activity is regulated by small molecules such as ATP and ADP (4–6), and the thyroid hormone T2 (7). COX contains skeletal muscle/heart (“heart-type”) and nonskeletal muscle (“liver-type”) isoforms of subunits VIa, VIIa, and VIII that have been known for the past 2 decades (reviewed in Ref. 8). We have recently discovered three additional isoforms: a lung-specific isoform of subunit IV, a third isoform of subunit VIII, and a testes-specific isoform of subunit VIb (9–11). Although it is clear that protein kinases and phosphatases are crucial in cellular signaling, little is known about their role in the regulation of the respiratory chain complexes.

There have been three studies of COX phosphorylation: Steenaert and Shore (12) examined mitochondrial proteins phosphorylated by endogenous kinases in the presence of [γ -³²P]ATP and identified COX subunit IV; Miyazaki *et al.* (13) showed that COX subunit II can be phosphorylated by nonreceptor tyrosine kinase c-Src in osteoblasts and found a positive correlation between COX activity and c-Src kinase activity (see also Ref. 14); and Bender and Kadenbach (15) incubated isolated COX from cow heart with commercially available protein kinase A (PKA), cAMP, [γ -³²P]ATP, and an ATP-regenerating system and observed the major signal for subunit Vb, along with signals for subunits II and/or III. The latter group later proposed that subunit I is also phosphorylated (16). These experiments demonstrate the possible modification of COX by serine/threonine-specific PKA *in vitro*, but no attempts were undertaken to show that PKA acts on COX *in vivo*.

We show here that cAMP-dependent COX phosphorylation occurs at subunit I under physiological conditions, mediated by an endogenous tyrosine kinase. Furthermore, we were able to assign cAMP-dependent phosphorylation to tyrosine 304 of COX subunit I and show that such phosphorylation leads to strong COX inhibition. An example of a function served by this mechanism is COX inhibition through the elevation of cAMP levels in liver cells triggered by glucagon during starvation periods, a condition where energy usage should be restricted.

EXPERIMENTAL PROCEDURES

Isolation of Cytochrome *c* Oxidase—Chemicals were purchased from Sigma unless otherwise stated. Mitochondria were isolated from cow liver and heart tissue as described (17) with several modifications. All steps were performed at 4 °C or on ice. Tissue (250 g) was ground and further homogenized with a commercial blender, applying a 5-fold volume of buffer A (250 mM sucrose, 20 mM Tris (pH 7.4), 2 mM EDTA). Importantly, this buffer was supplemented with 10 mM KF, 2 mM EGTA, and 10 mM theophylline to obtain phosphorylated COX, and the ground tissue was incubated with buffer A for 20 min at 4 °C before proceeding with the next step. The suspension was centrifuged (650 \times g, 10 min) and the supernatant was collected through cheesecloth. The pellet was homogenized and centrifuged one more time to increase the yield of mitochondria. Combined supernatants were centrifuged

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¹ The abbreviations used are: COX, cytochrome *c* oxidase; PKA, protein kinase A; AKAP, A-kinase-anchoring protein; HPLC, high pressure liquid chromatography; MS, mass spectrometry.

(14,000 $\times g$, 20 min). The mitochondria were washed by resuspending the pellet in 2.5 liters of buffer A using a Teflon homogenizer (150 rpm, 4 strokes) and centrifuged at low speed (300 $\times g$ for 5 min) to remove contaminants. The supernatant was centrifuged (14,000 $\times g$, 20 min) to collect mitochondria, which were washed two more times by resuspending and centrifuging (14,000 $\times g$, 20 min). The mitochondrial pellet was resuspended in buffer A and adjusted to a final protein concentration of 20 mg/ml. Per 1 ml of isolated mitochondria, 250 μ l of buffer B (1 M KH_2PO_4 , pH 7.4) were added. In order to solubilize mitochondrial proteins, 2 ml of 20% Triton X-114 per 1 g of heart mitochondrial protein were added dropwise with stirring. For liver mitochondria, only half the volume of detergent was added to prevent the extraction of COX. The suspension was centrifuged for 30 min at 195,000 $\times g$. The membrane fraction-containing pellet was resuspended with a Teflon homogenizer in 100 ml of buffer C (200 mM KH_2PO_4 , pH 7.4) and centrifuged for 25 min at 195,000 $\times g$. The pellet was resuspended in 50 ml of buffer D (200 mM KH_2PO_4 (pH 7.4), 5% Triton X-100) as above, applying three strokes and centrifuging for 25 min at 195,000 $\times g$. The pellet was resuspended in 100 ml of buffer C, applying 10 strokes and centrifuging for 20 min at 195,000 $\times g$. The COX-containing supernatant was collected, and COX was extracted from the pellet by repeating the previous step two times. Combined supernatants were diluted with 4 volumes of distilled H_2O and applied to 300 ml of DEAE-Sephacel column (fast flow; Amersham) equilibrated with buffer E (50 mM KH_2PO_4 (pH 7.4), 0.1% Triton X-100). The column was washed with 800 ml of buffer E, and proteins were eluted using a linear gradient from 50 mM to 1 M KH_2PO_4 (pH 7.4, 0.1% Triton X-100). COX usually elutes at an ionic strength of about 250 mM KH_2PO_4 (heart) or 300 mM KH_2PO_4 (liver). Eluted fractions were analyzed by spectrophotometer, and COX-containing fractions were combined. Sodium cholate was added (1% w/v) with stirring, and pH was monitored and adjusted to pH 7.4 with NaOH. Fractionations were performed with 28% ammonium sulfate for at least 6 h, 37% for at least 1 h, and 45% for 5 min; proteins were collected after each step by centrifugation for 15 min at 27,000 $\times g$. Precipitated proteins were dissolved in buffer F (50 mM KH_2PO_4 , pH 7.4) and stored at -80°C after determination of COX concentration (18) and purity (17) by spectrophotometer.

Preparation of Regulatory Competent COX and Enzymatic Activity Measurements—In order to obtain COX that shows regulatory properties, cholate, which is tightly bound (3) at nucleotide binding sites (5), has to be removed, and cardiolipin removed during COX isolation has to be replaced (19). Three μ M COX was resuspended in 0.5 mM ATP, 120 μ M cow heart cardiolipin, 50 mM KH_2PO_4 (pH 7.4), 1% Tween 20, 10 mM KF, and 2 mM EGTA. The suspension was dialyzed against the same buffer without cardiolipin for at least 14 h. COX activity was determined in a closed 200- μ l chamber containing a micro-Clark-type oxygen electrode (Oxygraph system; Hansatech). Measurements were performed at 25°C in 50 mM KH_2PO_4 (pH 7.4), 1% Tween 20, 10 mM KF, 2 mM EGTA, 20 mM ascorbic acid, and 5 mM nucleotides as indicated, using 150–300 nM COX and increasing amounts of cow cytochrome *c* from 0 to 40 μ M. All measurements performed in the presence of 5 mM ATP included an ATP-regenerating system as described (20). Oxygen consumption was monitored on a computer and calculated using the Oxygraph software. Turnover is defined as oxygen consumed (μM)/(s-COX (μM)).

COX Activity Measurements Using Cultured Cells or Frozen Tissue—HepG2 cells were grown to 95% confluence in modified minimum essential medium at 37°C and a 5% CO_2 atmosphere, supplemented with 10% fetal bovine serum, 1000 units of penicillin/streptomycin. Cells were washed with PBS, harvested by scraping in the presence of 10 ml of PBS, collected by centrifugation (50 $\times g$, 5 min), and washed again with PBS. The cell pellet was resuspended in incubation buffer (250 mM sucrose, 10 mM MgSO_4 , 20 mM K-HEPES (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, and 2 μ M oligomycin) and incubated in the presence or absence of glucagon (2 nM) or forskolin (5 μ M) at room temperature in the dark for 2 min or 20 min. Cells were collected by centrifugation, and 500 μ l of chilled solubilization buffer (10 mM K-HEPES (pH 7.4), 40 mM KCl, 1% Tween 20, 2 μ M oligomycin, 1 mM phenylmethylsulfonyl fluoride, 10 mM KF, 2 mM EGTA) were added, followed by cell disruption on ice with an ultrasonicator (micro tip, 4 \times 8-s pulses). Cell debris was removed by centrifugation (2 min, 16,000 $\times g$), and the supernatant was used for respiration measurements. Frozen tissue from cow liver or lung (50 mg) was partially disrupted in 500 μ l of incubation buffer using a Teflon microtube pestle applying 5 strokes (liver) or 10 strokes (lung), resulting in smaller cell clusters but leaving most cells intact. Cells were incubated and sonicated as described above. Protein concentration was determined with the DC protein assay kit (Bio-Rad).

Western Analysis—SDS-PAGE of COX was carried out as described (21). Protein transfer time on a nitrocellulose membrane was 45 min to

allow efficient transfer of the larger COX subunits. Anti-phosphoserine and anti-phosphothreonine antibodies are sets of four (1C8, 4A3, 4A9, and 16B4) and three (1E11, 4D11, and 14B3) individual monoclonal antibodies (Calbiochem), respectively, whereas a single anti-phosphotyrosine antibody was used (4G10; Upstate Biotechnology, Inc., Lake Placid, NY). Western analysis was performed with a 1:2000 dilution of anti-phospho antibodies, and signals were detected using horseradish peroxidase-conjugated secondary antibodies (ECL Western blotting detection kit; Amersham Biosciences).

ATP Assay—Frozen cow lung tissue (40 mg) was partially disrupted in 800 μ l of incubation buffer with a Teflon microtube pestle by applying 10 strokes. Fifty μ l of this cell suspension were transferred to each of eight tubes containing 50 μ l of incubation buffer, four tubes also containing theophylline to a final concentration of 0.1 mM. Cells were incubated at 4°C for 20 min followed by the addition of 275 μ l of boiling buffer (100 mM Tris (pH 7.75), 4 mM EDTA) and immediately transferred to a boiling water bath and lysed for 2 min. The tubes were centrifuged, and 5 μ l of the supernatant were utilized to determine the ATP concentration using the ATP bioluminescence assay kit HS II (Roche Applied Science) according to the manufacturer's protocol. Data were standardized to the protein concentration: 100 μ l of a 20% SDS solution were added to each boiled sample containing precipitated proteins and cell debris and ultrasonicated for a total time of 8 min. Protein concentration was determined as described above.

MS/MS Analysis of Bovine COX Preparation—A 5- μ g fraction of cow COX isolated in the presence of theophylline, KF, and EGTA (see above) was digested in 70% formic acid with 10% cyanogen bromide for 2 h in the dark. After digestion, peptides were diluted with three volumes of water and dried completely in a speedvac (Thermo Savant, San Jose, CA). Samples were then reconstituted in 0.1% acetic acid and introduced into an LCQ Deca quadrupole ion trap mass spectrometer (Thermo Electron, San Jose, CA) using a variation of the "vented column" approach as described (22). Peptides were loaded onto a pre-column (360- μ m outer diameter \times 75- μ m inner diameter; 2 cm of 5- μ m Monitor C18; Column Engineering, Ontario, CA) at a flow rate of 4 μ l/min with a Surveyor autosampler and HPLC pump (Thermo Electron). Peptides were eluted to the mass spectrometer using an analytical column (360- μ m outer diameter \times 75- μ m inner diameter; 12 cm of 5- μ m Monitor C18) and a linear gradient (0–50% B in 30 min, A = 0.1 M acetic acid, B = 0.1 M acetic acid in acetonitrile; flow rate ~200 nL/min). The mass spectrometer performed cycles of one MS scan followed by MS/MS scans (collision energy = 35%; 3-Da window; precursor $m/z \pm 1.5$ Da) of the three most abundant species in the initial MS scan. Dynamic exclusion was utilized with a repeat count of 1 and an exclusion time of 1.5 min.

Data Base Analysis—Tandem mass spectra were assigned to peptide sequences from the bovine NCBI nonredundant protein data base using Bioworks 3.1SR1 (Thermo Electron) and the SEQUEST algorithm (23). Search parameters specified a differential modification of +80 Da to serine, threonine, and tyrosine residues (phosphorylation) and –18 Da to methionine (homoserine lactone) and a static modification of –30 Da to methionine (homoserine). Phosphopeptide spectra were manually verified.

RESULTS

COX Shows cAMP-dependent Phosphorylation at Subunit I and Also Can Be Phosphorylated at Subunits II and IV—We have modified an established protocol for the isolation of COX from liver and heart tissue to obtain increased amounts of regulatory competent COX (see "Experimental Procedures"). For COX phosphorylation studies on the isolated enzyme, we included in all solutions during COX purification potassium fluoride, a nonspecific inhibitor of protein phosphatases, and EGTA, a calcium chelator, preventing activation of calcium-dependent protein phosphatases. Theophylline, a weak phosphodiesterase 4 inhibitor (24), was also included to increase cellular cAMP concentrations. We isolated liver COX side by side in the absence (referred to as "standard conditions") and presence of KF, EGTA, and theophylline. For comparison, we also isolated COX from heart tissue under standard conditions. COX subunits separated by SDS-PAGE and probed by Western analysis with monoclonal anti-phosphoserine, -threonine, and -tyrosine antibodies revealed three findings (Fig. 1): (a) COX subunit IV showed a strong signal in all three enzyme isola-

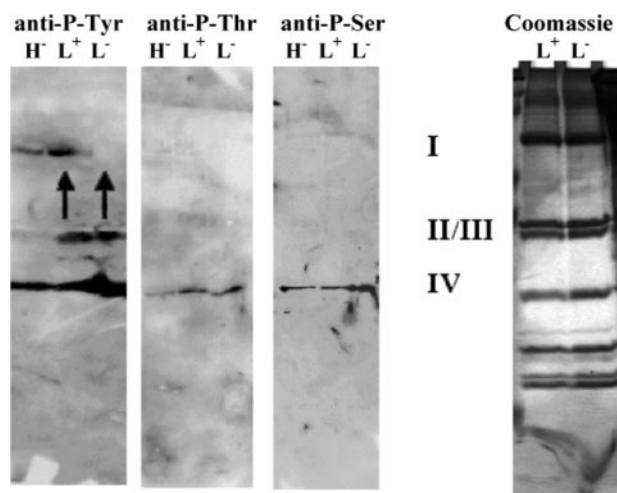


FIG. 1. Western blot analysis with anti-phospho antibodies of cow heart (H) and liver (L) COX. Cow liver COX was isolated side by side in the presence (+) or absence (–) of theophylline. COX subunits were separated by SDS-PAGE and transferred to a nitrocellulose membrane; blotting time was 45 min. which allows efficient transfer of the larger COX subunits I, II, III, and IV as indicated. Anti-phosphotyrosine (left blot), anti-phosphothreonine (middle blot), and anti-phosphoserine (right blot) antibodies were used (see “Experimental Procedures” for details). Subunit IV shows a signal with all three antibodies. Subunit II shows tyrosine phosphorylation if exposure time is increased (not shown). Subunit I of the liver enzyme, which was isolated in the presence of theophylline, shows a signal with the anti-phosphotyrosine antibody similar to heart COX, whereas liver COX isolated without theophylline shows no subunit I phosphorylation (arrows).

tions with the anti-phosphotyrosine antibody and weaker signals with both the anti-phosphoserine and -threonine antibodies; (b) liver subunit II reacted with the anti-phosphotyrosine antibody, in agreement with a recent report showing that subunit II can be phosphorylated (13); and (c) subunit I of the liver enzyme produced a strong signal with the anti-phosphotyrosine antibody when purified in the presence of theophylline, whereas no signal was observed in its absence (Fig. 1, arrows). Heart COX isolated in the absence of theophylline did reveal a weak signal with the anti-tyrosine antibody. Under the conditions applied for liver COX, only subunit I shows detectable differences in tyrosine phosphorylation upon increasing intracellular cAMP levels. No changes in serine or threonine phosphorylation were observed.

COX Subunit I Is Phosphorylated at Tyrosine 304—COX isolated in the presence of theophylline was digested with cyanogen bromide, which resulted in only the partial digestion of subunit I, with a sequence coverage of 6%. Based on analysis by nano-liquid chromatography/electrospray ionization/MS/MS, the fragment DVDTRAYFTSATM, which occurs in subunit I, was identified as containing a phosphate group on tyrosine 304 (Fig. 2). The phosphorylation site was unequivocally assigned by fragment ions b6, b7, b8, y6, y7, and y8. The sequence of the peptide was definitively assigned by b2, b5, b6, b7, b8, b9, b10, b11²⁺, b12²⁺, y3, y4, y5, y6, y7, and y8. Tyr³⁰⁴ is located on subunit I helix VIII, close to the intermembrane space (3). Helix VIII is in contact with the heme *a*₃ group, the reaction center where electrons are transferred to oxygen as their final acceptor. This helix also contacts helix II of catalytic subunit II (see “Discussion”).

COX Phosphorylated at Subunit I Is Inhibited and Shows Pronounced Allosteric Control—To investigate the functional implications of COX subunit I phosphorylation, we measured enzymatic activity by titrating COX with increasing amounts of cytochrome *c*. Measurements were performed in the presence of allosteric inhibitor ATP, activator ADP, or AMP. Liver COX

116.0	215.1	330.1	431.2	587.3	658.3	901.3	1048.4	1149.5	1236.5	1307.5	1408.6	1509.6	b
D	V	D	T	R	A	pY	F	T	S	A	T	M	
1509.6	1394.6	1295.5	1180.5	1079.5	923.4	852.3	609.3	462.2	361.2	274.1	203.1	102.1	y

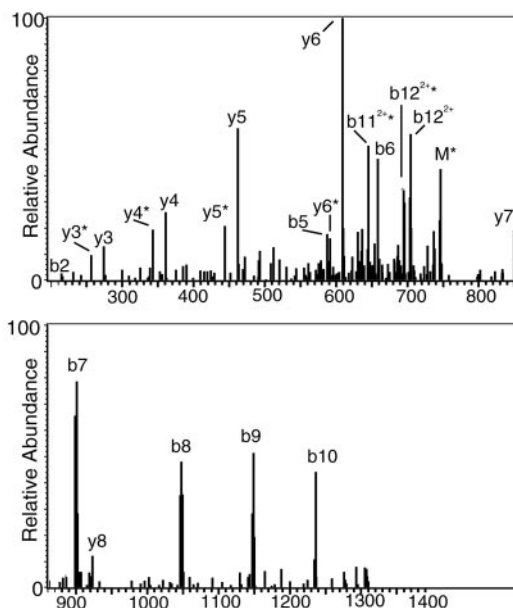


FIG. 2. Nano-liquid chromatography/electrospray ionization/MS/MS spectrum of DVDTRApYFTSATM. Peptides were eluted into the mass spectrometer with an HPLC gradient (0–50% acetonitrile, 0.1 M acetic acid in 30 min). The mass spectrometer acquired the top three data-dependent electrospray ionization MS/MS spectra. An asterisk indicates the neutral loss of water. The methionine residue was modified to homoserine lactone.

isolated in the presence (phosphorylated at subunit I) or absence (not phosphorylated at subunit I) of theophylline showed clear differences in kinetics (Fig. 3, Table I). First, V_{\max} of subunit I phosphorylated COX is lower compared with unphosphorylated COX. Second, enhancement of the allosteric response was seen in the phosphorylated COX, such that little or no activity is present below 10 μ M cytochrome *c* (Fig. 3A; see also Hill coefficients in Table I). Both AMP and ADP are seen to activate COX, but activation mediated by ADP is stronger.

Glucagon and Forskolin Treatment Leads to COX Inhibition—The peptide hormone glucagon acts as a strong physiological signal for increasing intracellular cAMP levels in liver by binding to G-protein-linked receptors, followed by activation of adenylyl cyclase (25). In order to relate our findings on the isolated enzyme to cellular physiology, we tested the effect of glucagon on COX activity with both intact human liver carcinoma HepG2 cells and cow liver tissue. We avoided artificially inducing the insulin pathway by scraping instead of trypsinizing HepG2 cells (26). Measurements using fed human HepG2 cells revealed strong inhibition of COX activity after treatment with glucagon (Fig. 4A). Similar results were obtained with cow liver tissue (not shown).

To determine whether actual starvation would cause the same effect on COX, we analyzed starved cells. These cells showed sigmoidal kinetics in the presence of the allosteric inhibitor ATP and the absence of glucagon (Fig. 4B). This tendency was further increased after preincubation with glucagon. Thus, we show for the first time that the glucagon receptor/G-protein/cAMP pathway strongly inactivates COX. Elevating intracellular cAMP levels by direct activation of adenylyl cyclase with forskolin revealed a similar effect on human HepG2 cells (Fig. 4C) and cow liver tissue (not shown) as observed with glucagon.

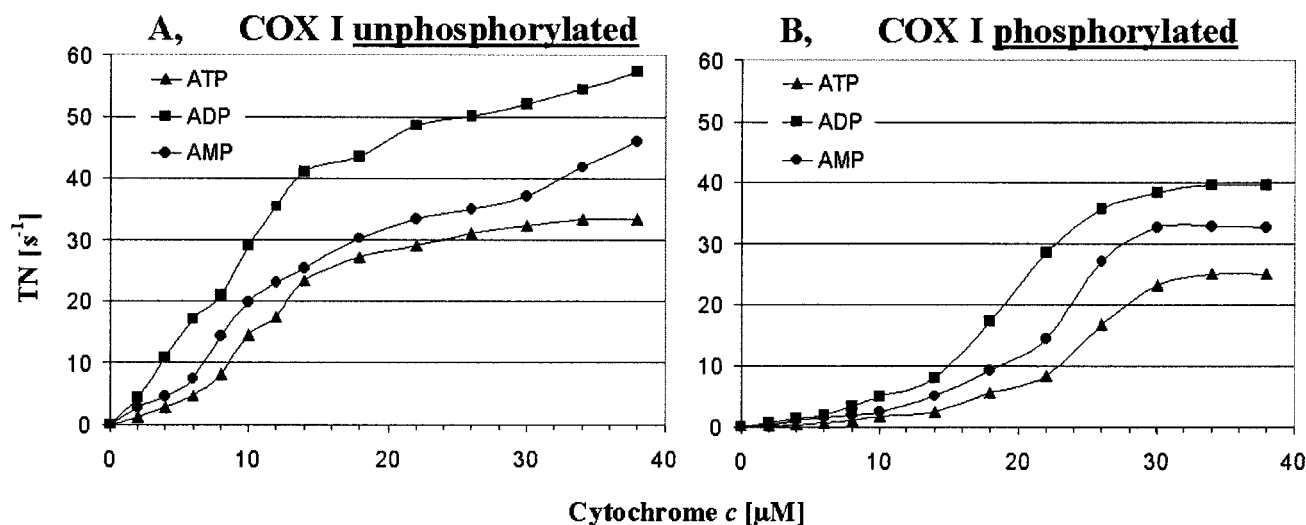


FIG. 3. Liver COX isolated in the presence or absence of theophylline shows different kinetic properties. The activity of solubilized COX (20), pretreated as described to restore regulatory properties, was measured in the presence of 20 mM potassium ascorbate, 5 mM ATP (▲), ADP (■), and AMP (●) at increasing concentration of cytochrome *c*. COX activity was measured with the polarographic method in a 200- μ l volume at 25 °C (54). COX activity (turnover; TN) is defined as follows: $V = \text{consumed } O_2 (\mu\text{M})/(\text{s} \cdot \text{COX } (\mu\text{M}))$.

TABLE I
Kinetic properties of cow liver COX isolated in the presence or absence of theophylline

	With theophylline				Without theophylline			
	Control ^a	ATP	ADP	AMP	Control ^a	ATP	ADP	AMP
K_m^b	22.5	23.7	18.8	23.0	10.5	12.4	9.5	10.9
V_{max}^b	25.8	25.0	39.6	32.7	38.1	33.4	55.0	46.0
Hill ^{b,c}	3.1	4.0	3.1	3.4	1.9	2.4	1.7	2.0

^a Enzyme after overnight dialysis with 0.5 mM ATP.

^b Kinetic parameters were calculated from data in Fig. 3.

^c The Hill coefficient was determined as described (29).

In order to test whether the cytosolic component of the cAMP signaling pathway is required for COX phosphorylation or whether cAMP can exert an effect on COX in isolated mitochondria, for example by directly activating a tyrosine kinase present in the intermembrane space, we incubated isolated mitochondria with cAMP as described (15). In contrast to a previous study (15), no differences in COX kinetics were observed using mitochondria isolated from fresh or frozen liver tissue in the presence or absence of cAMP (data not shown).

Theophylline Leads to COX Inactivation in the Lung—We tested the effect of the phosphodiesterase 4 inhibitor theophylline on cow lung tissue, applying a final concentration of 100 μ M, based on its use as an anti-asthma drug (24). Interestingly, even at these low concentrations, COX was inhibited, showing an enhanced sigmoidal tendency (Fig. 5A). In the presence of ADP and ATP, we observed a 25 and 60% inhibition at maximal turnover in the presence of theophylline. At low cytochrome *c* substrate concentrations, theophylline leads to almost full COX inhibition in the presence of ATP. We then assessed whether COX inhibition by theophylline leads to decreased energy levels. We found that ATP levels are significantly decreased (by 26%) under these conditions (Fig. 5B).

DISCUSSION

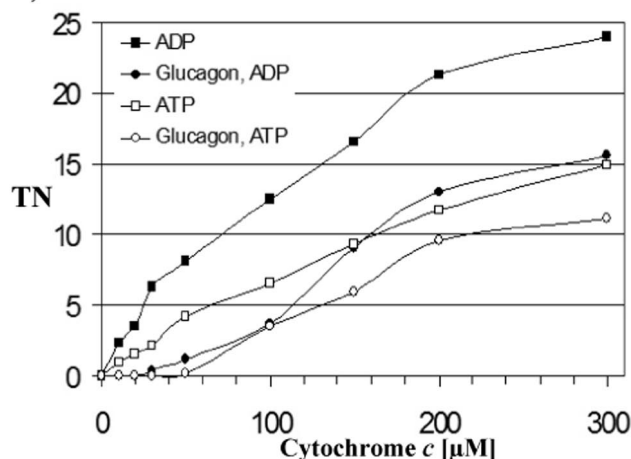
We show here cAMP-dependent COX phosphorylation of subunit I and its effect on COX activity. To do so, we combined enzyme activity measurements with mass spectrometry and Western blot analysis, all on enzyme preparations isolated by modifying previously used protocols. We were able to conclude that COX is switched off upon cAMP-dependent tyrosine phosphorylation of catalytic subunit I such that full or strong inhibition is present up to substrate concentrations of 10 μ M cyto-

chrome *c*, even in the presence of allosteric activator ADP. This novel mechanism, involving an endogenous mitochondrial tyrosine kinase, overrules COX regulation by allosteric effectors AMP, ADP, and ATP.

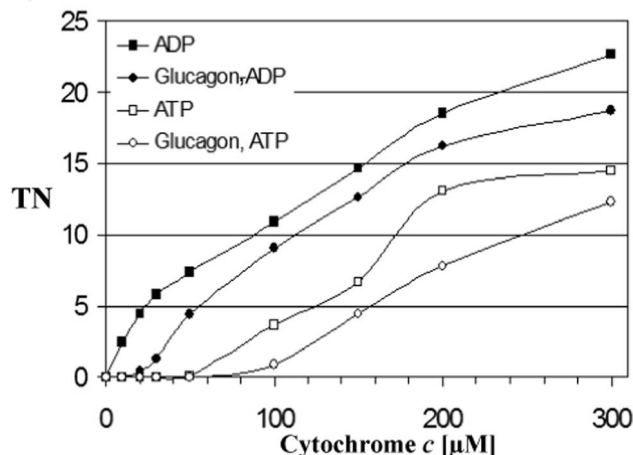
Our modifications of previously used protocols enabled us to isolate and analyze COX with regulatory properties. The major modifications were as follows. (a) Cholate, which is added as a detergent during COX isolation and binds strongly to COX, was removed by dialysis in the presence of nucleotides, which probably compete for the same binding sites (3, 5). (b) *N,N,N',N'*-Tetramethyl-*p*-phenylenediamine, which facilitates electron transfer between molecules, was not used in COX assays, since it prevents cooperativity. *N,N,N',N'*-tetramethyl-*p*-phenylenediamine-mediated electron transfer bypasses the association/dissociation reactions of cytochrome *c* and COX by reducing the COX-cytochrome *c* complex (27). (c) Dodecylmaltoside was avoided, since it has been suggested to monomerize COX (28) and thus prevent cooperativity (29). (d) Cardiolipin, which is important for COX function (30) and has been found in the crystal structure (31), was added in our assay. (e) Calcium, a frequently used buffer component during mitochondria isolation, was not used, and endogenous calcium was chelated with EGTA. Calcium is believed to be a key signal for mitochondrial activation (32), and it has been proposed to act on COX via activation of calcium-dependent phosphatases (15, 33). Our experiments support the presence of a calcium-dependent phosphatase, since calcium addition to isolated liver COX phosphorylated at subunit I is not sufficient to activate COX (data not shown). (f) Endogenous phosphorylation status was protected by the use of phosphatase inhibitors. The latter two points are especially important regarding respiration measurements, because signaling networks that act on COX are disrupted during isolation of mitochondria or COX, leaving mitochondrial kinases and phosphatases uncontrolled.

From COX isolated in the presence of theophylline, we have unambiguously identified COX subunit I residue Tyr³⁰⁴ in the intermembrane space as being phosphorylated (Fig. 2), which differs from the previously postulated cAMP-dependent phosphorylation of COX subunit I residue Ser⁴⁴¹ (16). Our finding suggests a novel signaling pathway involving a tyrosine kinase. Most signals conveyed by cAMP are mediated through phosphorylation reactions catalyzed by serine/threonine-specific PKA (34), and several studies have suggested that PKA activity

A, fed cells



B, starved cells



C

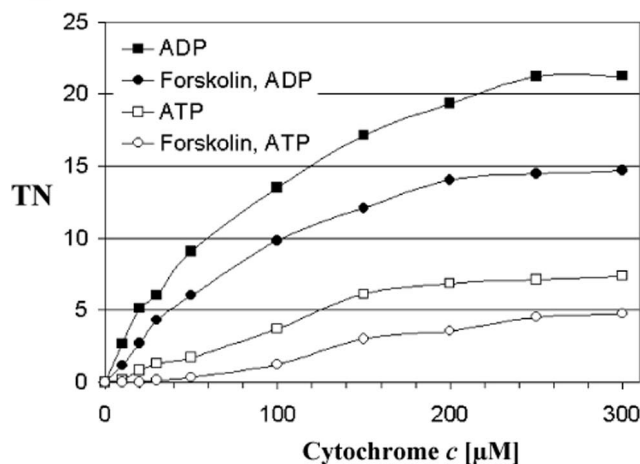
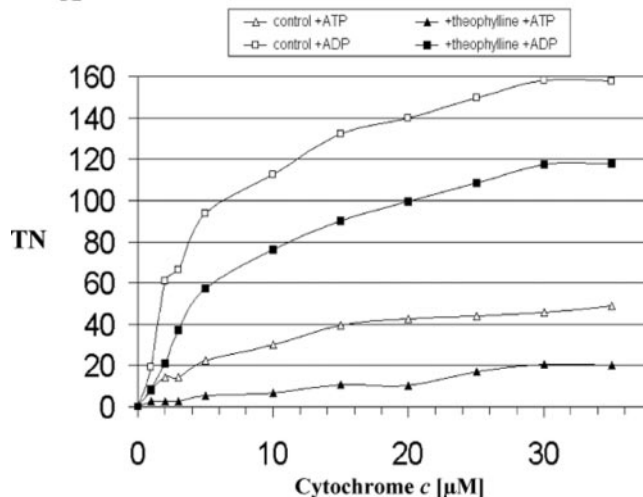


FIG. 4. **Effect of glucagon and forskolin on COX activity.** A, HepG2 cells were grown to 95% confluence. Fresh medium containing 0.1% glucose was added, and cells were incubated for 6 h. These fed HepG2 cells were resuspended in incubation buffer in the presence (circles) or absence (squares) of 2 nM glucagon and incubated at 20 °C for 2 min. Samples were immediately used for COX activity measurement in the presence of 5 mM ATP (open symbols, inhibited) or ADP (filled symbols, stimulated). Higher amounts of cow cytochrome *c* necessary to stimulate human COX compared with cow COX result from the imperfect match (55). Turnover (TN) is defined as consumed O_2 (μM)/(min-total protein (mg)). B, HepG2 cells were incubated for 48 h without medium change. Starved cells were incubated in the presence or absence of glucagon as above. C, fed HepG2 cells were incubated in the presence or absence of 5 μM forskolin for 20 min.

A



B

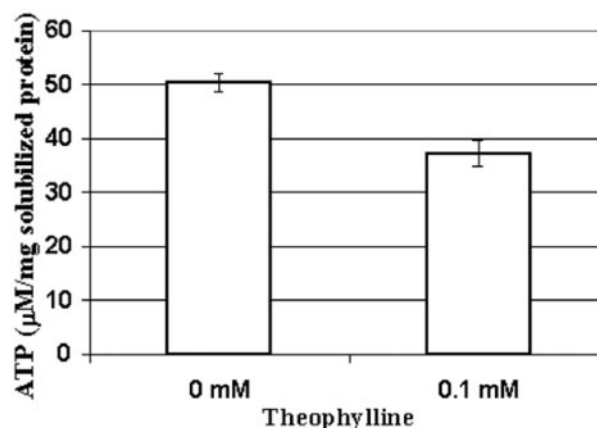


FIG. 5. **Incubation of cow lung tissue with theophylline leads to COX inhibition and decreased ATP levels.** A, cow lung tissue was incubated at 4 °C for 20 min in the presence (filled symbols) or absence (open symbols) of 100 μM theophylline. COX activity was determined in the presence of 5 mM ATP (triangles) or ADP (squares) as described in the legend to Fig. 3 after disruption of the cells via ultrasonication. Turnover (TN) = consumed O_2 (μM)/(min-total protein (mg)). B, ATP concentrations were measured using the bioluminescence method.

is present in mitochondria of yeast, cell lines, and mammalian tissues (35–41, 42, 43). However, central for PKA-mediated phosphorylation under physiological conditions are protein kinase A-anchoring proteins (AKAPs), which localize PKA to cellular compartments in close proximity to its targets (44); S-AKAP84 and AKAP121 have been shown to mediate localization of PKA to the outer side of the outer mitochondrial membrane and to be crucial for cAMP-dependent signaling to the mitochondria (45–47). Also, in this study we found that incubation of isolated mitochondria with cAMP did not change COX activity. These findings are consistent with our proposed signal transduction pathway, in which PKA does not act directly on COX but rather conveys a signal through the outer mitochondrial membrane that leads to activation of a tyrosine kinase present in the intermembrane space. A considerable number of tyrosine kinase candidates are available; more than 100 have been predicted (48), for most of which the cellular localization or function remains to be elucidated.

The effect of subunit I phosphorylation on liver COX activity is displayed in Figs. 3–5. The operational definition of subunit I phosphorylation is isolation in the presence of theophylline,

since probing the separated subunits with anti-phospho antibodies shows this to be the only variable detectable. Stimulation by ADP of the unphosphorylated enzyme follows a saturation curve that remains linear to about 12 μM cytochrome *c*, where activity is >70% of that at 40 μM cytochrome *c* (Fig. 3). By contrast, the phosphorylated enzyme shows sigmoidal behavior such that activity at 12 μM cytochrome *c* is less than 20% compared with saturating substrate concentrations. Thus, substantial allosteric regulation of activity by reversible phosphorylation could take place at the substrate concentrations of 6 μM cytochrome *c* reported for normal mitochondrial heart muscle particles (49). Similar effects were observed in preparations inhibited or stimulated with ATP and AMP, respectively (Fig. 3, Table I). Based on the activity of subunit I phosphorylated COX, we conclude that our isolation conditions yield an enzyme that is phosphorylated on one or both subunits I per dimer, because no respiration was apparent at low cytochrome *c* concentrations, whereas subunit I unphosphorylated COX was active already at very low cytochrome *c* concentrations (Fig. 3).

Other treatments that would increase phosphorylation were tested in a cell culture system and found to have the effect on activity and cytochrome *c* concentration dependence predicted by the theophylline results. This is seen when cAMP levels are raised by glucagon addition (Fig. 4, A and B) or activation of adenyl cyclase with forskolin (Fig. 4C). Glucagon-promoted COX inhibition is physiologically consistent, since under food

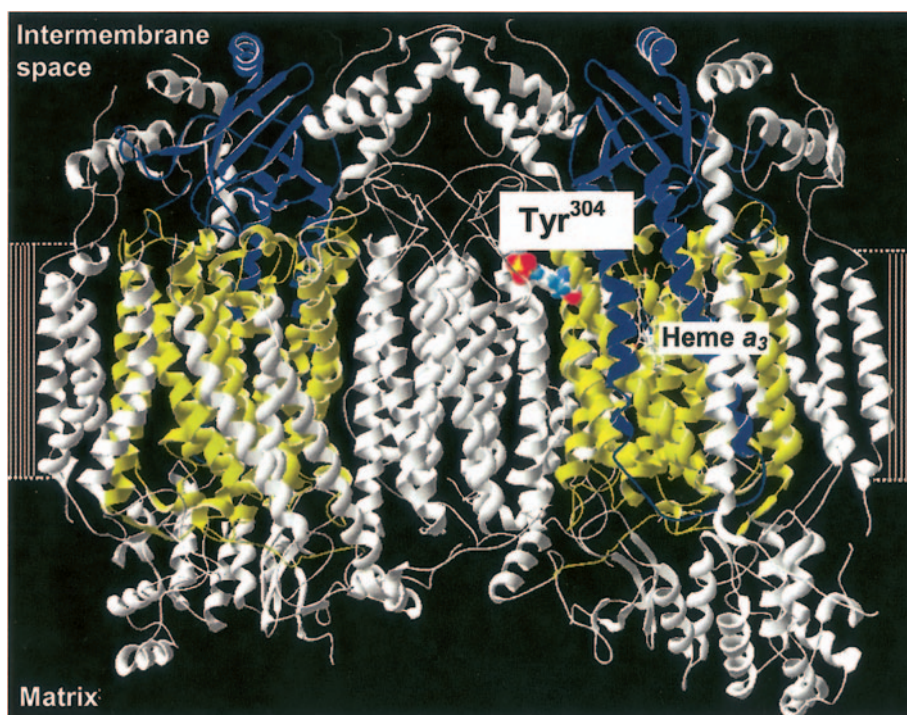
deprivation conditions energy usage should be restricted and, in periods of starvation, such as during sleep, glucagon, the most potent hormone increasing intracellular cAMP levels in liver cells, is released from the pancreatic α -cells. Previous attempts to detect effects of glucagon on COX activity in hepatocytes revealed no significant effects (e.g. Ref. 50), most likely because the phosphorylation status was not preserved due to the presence of calcium and absence of phosphatase inhibitors in the isolation or incubation buffers. In addition, differences in enzyme kinetics might have been missed, since most studies have only determined V_{max} . The differences in V_{max} (>30 μM cytochrome *c*) for subunit I unphosphorylated COX are between 32 and 52% higher compared with phosphorylated COX (Table I), whereas the effect at a physiologically more relevant cytochrome *c* concentration of 6 μM (49) are dramatic, since COX is switched off when phosphorylated.

Tyrosine 304 of subunit I is conserved in all eukaryotes that we have examined and is present in some bacteria (Fig. 6). Bacteria lack hormonal signaling cascades, although cAMP is known to be a potent signal in bacteria. Thus, it will be interesting to determine whether the analogous prokaryotic tyrosine residue can be phosphorylated. Tyrosine 304 is located close to the heme a_3 -Cu_B reaction center, on a helix that is in contact with the other catalytic subunit, subunit II, and facing the interface region of the two COX monomers (Fig. 7). Consequently, the effect of phosphorylation is plausible. The Hill coefficient, which can be interpreted as a value for cooperativity of substrate binding sites, is clearly increased in the case of subunit I phosphorylated COX (Table I), which shows sigmoidal reaction kinetics (Fig. 3). Phosphorylation of Tyr³⁰⁴ at the interface of the two monomers might therefore enhance the monomer-monomer interaction. For dimeric COX with only one cytochrome *c* binding site per monomer, one would expect Hill values not higher than 2. Interestingly, we obtained higher values for the subunit I phosphorylated COX (3.1–4.0) compared with values around 2 for COX not phosphorylated at subunit I. Our higher Hill values would be consistent with the presence of a high and a low affinity cytochrome *c* binding site per monomer (51, 52), for which there could be sufficient space

Species	Seq. start	Sequence	Prot. acc. #
Cow	298	DVDTRAYETSATM	AAS18244
Zebrafish	298	DVDTRAYETSATM	AAF74299
<i>Drosophila melanogaster</i>	297	DVDTRAYETSATM	AAF77245
<i>Caenorhabditis elegans</i>	305	DLDSRAYFSATM	NP_006961
Rice	300	DVDTRAYETAATM	ODR21
<i>Saccharomyces cerevisiae</i>	298	DADTRAYETSATM	AJ011856
<i>Paracoccus denitrificans</i>	330	SLTQQTFFQMATM	P08305
<i>Rhodobacter spheroides</i>	341	SLTQQSYFMATM	P33517
<i>Bacillus subtilis</i>	306	GPIANALFAVATM	P24010
<i>Thermus thermophilus</i>	291	DPTWKMLHSVLTL	Q56408

FIG. 6. **Sequence alignment of cytochrome *c* oxidase subunit I.** COX I sequences were aligned with the program MegAlign. Selected eukaryotic (upper part) and prokaryotic representatives (lower part) are shown. The phosphorylation site (boxed) is conserved in more than 100 eukaryotes analyzed but not conserved in prokaryotes.

FIG. 7. **Cytochrome *c* oxidase crystal structure from cow heart.** Crystallographic data (31) were processed with the program Swiss PDB viewer. Shown is a side view of dimeric cytochrome *c* oxidase. The membrane region is indicated by dotted lines. Catalytic subunits I and II are highlighted in yellow and blue ribbons, respectively. The introduction of the phosphate group to Tyr³⁰⁴ to the right monomer and the rotation of the phosphotyrosine side chain, which in the crystal structure points to the membrane center, was performed using the program Hyperchem. A geometrically optimized structure for the phosphotyrosine side chain was obtained applying 500 cycles of the "steepest descent" algorithm, producing a conformation (highlighted in space fill) that is accessible from the intermembrane space. Tyr³⁰⁴ is located on helix VIII, which contacts catalytic subunit II and the heme a_3 -Cu_B reaction center (shown in sticks). The modeled phosphorylated Tyr³⁰⁴ points to the left monomer.



according to computer modeling (53).²

An interesting sidelight was examination of theophylline in the context of its use as an anti-asthma drug, for which the molecular mechanism remains to be clarified (24). Theophylline leads to COX inhibition in the lung at a similar concentration to that used in therapy. Concomitantly, ATP levels are significantly decreased (Fig. 5B). Since airway constriction during asthma requires energy, theophylline-promoted COX subunit I phosphorylation, which leads to inhibition of COX and therefore aerobic metabolism, could represent at least a component of theophylline action. If this explanation is correct, the observed effect would be expected to be more profound when substrates for the glycolytic pathway become limited.

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² V. A. Roberts, personal communication.

cAMP-dependent Tyrosine Phosphorylation of Subunit I Inhibits Cytochrome *c* Oxidase Activity

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