THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 13, Issue of March 29, pp. 11321–11328, 2002
Printed in U.S.A.

Chronic Treatment with Azide *in Situ* Leads to an Irreversible Loss of Cytochrome *c* Oxidase Activity via Holoenzyme Dissociation*

Received for publication, December 21, 2001
Published, JBC Papers in Press, January 16, 2002, DOI 10.1074/jbc.M112303200

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Chronic treatment of cultured cells with very low levels of azide (I_{50}<10 μM) leads to slow ($t_{1/2}$ = 6 h), irreversible loss of cytochrome *c* oxidase (COX) activity. Azide-mediated COX losses were not accompanied by inhibition of other mitochondrial enzymes and were not dependent upon electron flux through oxidative phosphorylation. Although azide treatment also reduced activity (but not content) of both CuZn superoxide dismutase and catalase, a spectrum of pro-oxidants (and anti-oxidants) failed to mimic (or prevent) azide effects, arguing that losses in COX activity were not due to resultant compromises in free radical scavenging. Loss of COX activity was not attributable to reduced rates of mitochondrial protein synthesis or declines in either COX subunit mRNA or protein levels (COX I, II, IV). Co-incubation experiments using copper ($CuCl_2$, Cu-His) and copper chelators (neocuproine, bathocuproine) indicated that azide effects were not mediated by interactions with either Cu_A or Cu_B. In contrast, difference spectroscopy and high performance liquid chromatography analyses demonstrated azide-induced losses in cytochrome aa_3 content although not to the same extent as catalytic activity. Differential azide effects on COX content relative to COX activity were confirmed using a refined inhibition time course in combination with blue native electrophoresis, and established that holoenzyme dissociation occurs subsequent to losses in catalytic activity. Collectively, these data suggest that COX deficiency can arise through enhanced holoenzyme dissociation, possibly through interactions with the structure or coordination of its heme moieties.

Most ATP required by eukaryotic cells under resting conditions is generated aerobically by mitochondrial oxidative phosphorylation (OXPHOS).[1] Maintenance of appropriate stoichiometries of OXPHOS complexes is critical not only to ensure efficient flux of electrons through the ETC but also to minimize the potentially cytotoxic impact of mitochondrially derived ROS (1, 2). Declines in the content of individual OXPHOS complexes frequently accompany cardiovascular and neurological diseases, as well as aging (3, 4).

The origins and consequences of the loss of a given OXPHOS complex are frequently studied in cell lines established from patients with metabolic deficiency (5–7). However, the pathophysiological consequences of complex deficiencies have also been studied using specific inhibitors to chronically impair catalytic activity (2, 8). Sodium azide is commonly used *in vitro* as a rapid and reversible inhibitor of COX, the terminal enzyme of the ETC that catalyzes the transfer of electrons from reduced cytochrome *c* to molecular oxygen (9–11). The ability of azide to rapidly bind to the binuclear center, thereby inhibiting COX activity, has led to its frequent use in acute studies, including those designed to address the role of mitochondrial oxygen sensing in signal transduction (12, 13). We previously reported that chronic treatment of cultured myocytes with micromolar levels of azide results in the irreversible inhibition of COX activity (14). This mode of inhibition clearly differs from the classic mechanism of azide-mediated COX inhibition in that it is of higher affinity (0.01–0.1 *versus* 1–10 mM) and irreversible in nature. The mechanism(s) by which azide mediates these effects is unknown. Moreover, its potential relevance to COX deficiency observed in a more physiologically relevant context has not been evaluated despite previous reports of selective COX losses leading to Alzheimer's disease-like symptoms in rats that had been chronically infused with sodium azide (15, 16).

Several plausible mechanisms exist that could account for the azide-mediated, irreversible inhibition of COX activity. First, the capacity of azide to act as a chelator of first order transition series metals could affect COX activity by either terminal binding to or stripping of one or both copper centers from highly conserved domains within mitochondrially encoded subunits I and II (Cu_A on COX II, Cu_B on COX I; Refs. 11 and 17). Second, because copper metabolism is critical to the maturation and assembly of individual COX subunits into a functional holoenzyme complex (18–20), azide may impair either its delivery or insertion into the complex. Third, azide may act as a suicide metabolite to inhibit COX activity during enzyme

* This work was supported through grants-in-aid of research from the Heart and Stroke Foundation of Canada (to C. D. M.) and Canadian Institutes of Health Research (to D. M. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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** Supported by a Natural Sciences and Engineering Research Council Fellowship.

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[1] The abbreviations used are: OXPHOS, oxidative phosphorylation; ASMC, aortic smooth muscle cells; COX, cytochrome *c* oxidase; CS, citrate synthase; Cu-His, copper histidine; ETC, electron transport chain; HEK293, human embryonic kidney 293 cells; HS, horse serum; $ONOO^-$, peroxynitrite; $O_2^{\cdot-}$, superoxide anion; PBS, phosphate-buffered saline; PC12, pheochromocytoma 12 cells; ROS, reactive oxygen species; HPLC, high performance liquid chromatography; SOD, superoxide dismutase; SSC, standard saline citrate; DCF, 2,7-dichlorofluorescin.

turnover in a manner analogous to azide-mediated loss of catalase activity (21). Accordingly, it has recently been shown that the COX-mediated conversion of azide to the azidyl radical leads to its irreversible inactivation *in vitro*, although this mechanism requires very high concentrations of both azide and H_2O_2 (22). Fourth, chronic azide effects on COX activity may arise indirectly from elevated bulk phase production of ROS since azide is also a potent inhibitor of SOD (23) and catalase (21, 24) activities.

In the present study, we systematically addressed these possibilities to clarify the mechanism(s) by which azide irreversibly inhibits COX activity. Collectively, our studies suggest that azide treatment results in a loss of catalytic activity by accelerating the rate of holoenzyme dissociation, possibly through interactions with the structure or coordination of heme moieties.

EXPERIMENTAL PROCEDURES

Cell Culture—All culture media, sera, and antibiotics were purchased from Invitrogen. C2C12 cells were grown in proliferation medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. At 70% confluency, myoblasts were switched to differentiation medium consisting of Dulbecco's modified Eagle's medium supplemented with 2% HS. Sol8, HEK293, and ASMC were cultured in proliferation medium supplemented with 10% fetal bovine serum, whereas PC12 cells were maintained in proliferation medium containing 20% fetal bovine serum. Penicillin, streptomycin, and neomycin were included in all media. The medium was changed every 2–3 days in proliferating and serum-starved cells.

Enzyme Assays, Metabolite Analyses, and Respiration Measurements—Assays for all enzymes were optimized to ensure that neither substrates nor co-factors were limiting. The activities of NADH ubiquinone oxidoreductase (EC 1.6.5.3), COX (EC 1.9.3.1), CS (EC 4.1.3.7) (25), glutathione peroxidase (EC 1.11.1.9) (26), and catalase (EC 1.11.1.6) (27) were measured from whole cell extracts as previously described. The activities of individual ETC enzymes were also assayed using isolated mitochondria (28). The complex V (EC 3.6.1.34) assay contained 5 mM $MgCl_2$, 100 mM KCl, 1 mM phosphoenolpyruvate, 5 mM ATP, 0.15 mM NADH, and 1 unit each lactate dehydrogenase and pyruvate kinase in 50 mM HEPES (pH 7.4) in the presence or absence of saturating amounts of oligomycin (0.24–0.72 μg). No NADH oxidation was observed in the absence of mitochondria. Total cellular SOD (EC 1.15.1.1) was assayed at 550 nm using the aerobic xanthine/xanthine oxidase system (23, 29). MnSOD activity was measured from mitochondrial extracts in the presence of 5 mM KCN to inhibit any contaminating CuZn SOD. Protocols for measuring cellular protein content, lactate concentration in the culture media, and whole cell respiration were as previously described (16).

Fluorescence Microscopy—Subconfluent myoblasts were grown in 10% fetal bovine serum on poly-L-lysine-treated glass coverslips. Coverslips were fitted to a 260-μl perfusion chamber thermostatted to 37 °C (Warner Instruments) on a Zeiss fluorescent microscope stage, superfused for 35 min with PBS supplemented with 25 mM glucose, 4 mM glutamine, 1 mM pyruvate, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 1% penicillin-streptomycin-neomycin containing 5 μM 2,7-dichlorofluorescin (DCF) diacetate (Molecular Probes). Fluorescence was measured (20× magnification, 100-ms exposure, 4 × 4 binning) using a CCD camera (Cooke Sensicam) and optical filters appropriate for DCF fluorescence (excitation peak, 490 nm; emission peak, 526 nm). The same cells were subsequently perfused for 5 min with 5 mM H_2O_2 to obtain maximal fluorescence (30). The mean pixel intensities of cells and background were determined using Slidebook (Intelligent Imaging Innovations). Because of potential excitation-dependent oxidation of DCFH, only one field of view was analyzed on each coverslip.

Electrophoresis and Immunoblotting—Plates were rinsed and harvested in ice-cold PBS. Pelleted cells were flash-frozen in liquid nitrogen and stored at −80 °C for at least 24 h. Thawed cells pellets were resuspended in ice-cold isolation buffer (250 mM sucrose, 20 mM HEPES (pH 7.4), and 1 mM EDTA) supplemented with a protease inhibitor mixture (Sigma) and homogenized with 10 passes through a pre-chilled, zero clearance homogenizer (Kontes Glass Co.). Cell debris and nuclei were pelleted with 2 10-min spins at 650 × *g*. The resultant supernatant (S1) was spun for 15 min at 14,000 × *g* to collect the mitochondrial fraction.

Denatured mitochondrial and S1 fractions (10 μg) were resolved using a 12% SDS-PAGE gel (31), and electroblotted (Bio-Rad, Miniprotean system) onto nitrocellulose membranes (Zymotech Inc.). Rabbit polyclonal antibodies directed against Cu/Zn and MnSOD (StressGen Biotechnologies Corp.) and cytochrome *c* (Santa Cruz Biotechnologies) and mouse monoclonal antibodies for anti-COX I, COX IV, and ND1 (Molecular Probes) were diluted to 1:1,000. Polyclonal anti-COX II (Dr. T. L. Mason, University of Massachusetts), anti-actin (Sigma), and anti-catalase (Rockland Immunochemicals Inc.) antibodies were diluted to 1:40, 1:5,000, and 1:25,000, respectively. Mouse anti-39-kDa (Molecular Probes) and human anti-porin (Calbiochem) monoclonal antibodies were diluted to 1:2,000. COX I and IV antibodies were resuspended in PBS containing 1% bovine serum albumin and 0.1% Tween 20. All other antibodies were reconstituted in Tris-buffered saline containing 0.05% Tween 20 supplemented with 2% low fat skim milk powder. Membranes were blocked for 1 h at room temperature in Tris-buffered saline/Tween supplemented with 5% low fat skim milk and incubated overnight at 4 °C in the primary antibody of interest. After a 1-h incubation at room temperature with either secondary anti-mouse (Pierce, 1:10,000) or anti-rabbit (Promega, 1:2,500) horseradish peroxidase antibodies, immunoreactive proteins were detected by luminol-enhanced chemiluminescence (Pierce).

Blue native PAGE (32, 33) was performed on mitoplasts that were isolated using a 1.2:1 digitonin to protein ratio and solubilized (1.5 mg/ml) on ice for 30 min in 750 mM 6-aminocaproic acid, 50 mM Bistris (pH 7.0), 1.5 mM EDTA, and 1.5% w/v laurylmaltoside (34). Samples were centrifuged for 20 min at 20,000 × *g*, and the relative distribution of complex I activity between the pellet and the extract was measured as an index of solubilization of inner membrane proteins (extract typically contained 80–90% of the total activity). Loading dye was added to each sample at a 1:4 ratio of Coomassie:laurylmaltoside, and equal units of complex I were loaded in each lane of a 6–15% continuous gradient gel. Protocols used for in-gel assays (35), immunoblotting of first-dimension gels and second dimension electrophoresis (36) were as previously described. NADH ubiquinone oxidoreductase and COX holoenzyme levels from first-dimension immunoblots were quantified using an enhanced chemifluorescence detection system (Amersham Biosciences, Inc.) according to the manufacturers' specifications.

Heme Analyses—Mitochondria (2.5 mg/ml) were solubilized on ice for 1 h in 750 mM 6-aminocaproic acid, 50 mM Bistris (pH 7.0) containing 1% w/v Triton X-100. Samples were centrifuged for 10 min at 14,000 × *g* to remove debris. UV-visible spectra were recorded on an OLIS-refurbished Aminco DW-2 UV/VIS spectrophotometer at room temperature and are presented as reduced-oxidized differences. The oxidized state is taken as the form of the sample in air and is unchanged upon the addition of ferricyanide. The reduced state is generated by addition of a few grains of solid sodium dithionite to the air-oxidized sample. Total heme A content from 3 mg of mitochondrial protein was extracted and measured by reverse phase HPLC as previously described (37).

RNA Isolation and Northern Analysis—Total RNA was purified from guanidinium thiocyanate extracts using a standard acid phenol protocol (38). Denatured RNA (10 μg) was fractionated using a 1% agarose-formaldehyde gel system. A cDNA for the muscle-specific isoform of COX VIa was amplified from rat soleus reverse-transcriptase template at 60 °C with 5′-ctgacctttgtgctggctct-3′ and 5′-gattgacgtggggattgtg-3′ using standard PCR conditions, cloned into pCR 2.1 (Invitrogen), and sequenced. All other probes for mtDNA- and nuclear-encoded mRNA species were obtained as previously described (16, 25).

Membranes were prehybridized (3 h) and hybridized (12–18 h) at 65 °C in modified Church's buffer (0.5 M sodium phosphate (pH 7.0), 7% SDS, and 10 mM EDTA). Membranes were washed twice at room temperature for 15 min with 2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), 0.1% SDS and twice at 50 °C for 15 min with 0.1× SSC, 0.1% SDS. Blots were visualized by phosphorimaging, and relative signal strength was quantified using ImageQuant software (Molecular Dynamics). Differences in loading across lanes were normalized using a probe for α-tubulin mRNA.

Pulse Labeling Experiments—To pulse label mitochondrial translation products (39), myotubes grown in 60-mm plates were washed twice with sterile PBS and incubated for 30 min in pre-warmed, methionine-free Dulbecco's modified Eagle's medium supplemented with 2% dialyzed HS. After an additional 5-min incubation in the presence of 100 μg/ml emetine, cells were incubated with 400 μCi of Expre^{35}S^{35}S (Mandel) for 1 h. Excess label was chased by the addition of regular Dulbecco's modified Eagle's medium, 2% HS for 10 min. Cells were washed 3 times with PBS and harvested by trypsinization. Samples (10 μg) were sonicated on ice and fractionated on a 12–20% gradient gel at 7 mA for 14–16 h. The gel was then fixed for 1 h and dried for autoradiography.

Statistical Analyses—Significant differences ($p < 0.05$) between control and treated groups for all measured parameters were detected

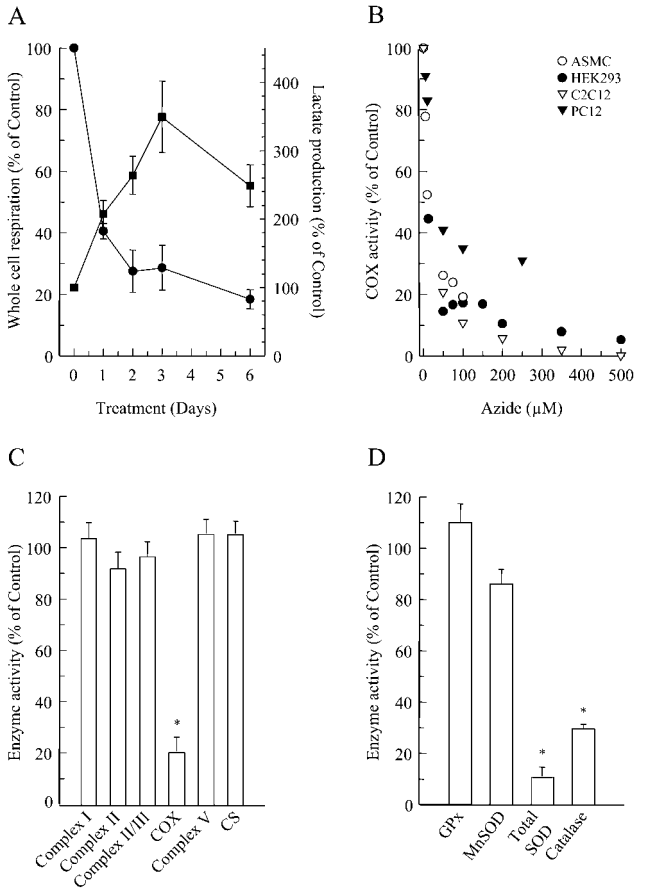

FIG. 1. **Chronic treatment of cultured cells with azide results in loss of COX activity, bioenergetic deficit, and altered cellular capacity for ROS metabolism.** *A*, whole cell respiration (●) and lactate production (■) in day-6 serum-starved C2C12 myotubes treated with 50 μM azide for 6 days (mean ± S.E., $n = 6$). *B*, titration of COX activity in HEK293, ASMC, PC12, and C2C12 cells treated with increasing concentrations of azide (0–500 μM) for 24 h. Rates ($\mu mol \cdot min^{-1} \cdot mg^{-1}$) in controls were 16.6 (ASMC), 12.1 (HEK293), 212.2 (PC12), and 105.3 (C2C12). *C*, activities of bioenergetic enzymes assayed from isolated mitochondria. *D*, antioxidant enzymes measured from whole cell extracts of day-6 serum-starved C2C12 myocytes treated with 100 μM azide for 24 h (mean ± S.E., $n = 3$). All rates are expressed relative to controls. The *asterisk* denotes a significant difference ($p < 0.05$) between control and azide-treated cells.

using one-way analysis of variance and identified *post hoc* using the Tukey-Kramer honestly signficantly different.

RESULTS

Chronic Azide Treatment Results in an Irreversible Loss of COX Activity, a Concomitant Decline in Whole Cell Respiration, and a Resultant Bioenergetic Stress—Chronic treatment of C2C12 myotubes with micromolar amounts of azide caused a time-dependent loss of COX activity (data not shown; Ref. 14), a resultant decline in whole cell respiration, and a concomitant increase in the rate of intracellular lactate accumulation (Fig. 1*A*). The azide effect on COX activity was also observed in three other cell backgrounds (HEK293, PC12, and ASMC), with a 24-h treatment with 100 μM azide, resulting in the inhibition of 65–90% of total activity (Fig. 1*B*).

Loss of catalytic activity was irreversible, both *in situ* and *in vitro*. C2C12 myotubes pulsed for 6 h with 100 μM azide continued to lose COX activity 24 h after the removal of azide from the culture media (6 *versus* 24 h, 49 *versus* 33% of control). Azide-treated C2C12 cell extracts dialyzed for 24 h (50 mM Tris (pH 8.0), 4 °C) in the presence or absence of reductant (β-mercaptoethanol, dithiothreitol) also failed to recover catalytic activity (data not shown).

To determine whether azide required access to the binuclear center to irreversibly inhibit COX activity *in situ*, C2C12 myotubes were co-incubated with equimolar amounts of azide and cyanide (10 μM each). Despite a much higher relative affinity of cyanide (K_i 1 *versus* 64 μM; Refs. 10 and 41) and its ability to competitively displace bound azide from the binuclear center *in vitro* (42), it neither attenuated nor abrogated azide-mediated COX inhibition (expressed as % of control: azide, 51.1; KCN, 102.5; azide + KCN, 47.1). Collectively, these data argue against azide exerting its *in situ* effects on COX activity via classically described ligand interactions with the binuclear center (see Refs. 9 and 10).

Chronic Azide Effects on Mitochondrial Enzymes Are Specific to COX and Do Not Require a Functionally Intact Respiratory Chain—The irreversible loss of catalytic activity could not be mimicked by dialyzing extracts of untreated C2C12 myotubes, rat skeletal muscle, or purified bovine COX against 0–100 μM azide (24 h in 50 mM Tris (pH 8.0), 4 °C; data not shown). Given that azide-induced losses in catalytic activity only occurred *in situ*, we investigated the potential requirement of an intact ETC to the observed azide effects (see Ref. 42). Previous reports that azide is a potent inhibitor of the ATPase activity of complex V (43, 44) also prompted us to evaluate its effects on the activity of a spectrum of other mitochondrial enzymes. Although azide treatment resulted in a significant loss of COX activity, it had no effect on a Krebs cycle enzyme (CS), individual complexes of the ETC, and the ATPase component of complex V (Fig. 1*C*). Incubation of C2C12 cells for 24 h with specific inhibitors of complexes I (2–200 nM rotenone) and III (2 μg/ml antimycin A, 0.1–10 μM myxothiazol) in the presence or absence of azide neither caused a specific COX loss nor abrogated azide-mediated COX losses (data not shown). Although 24-h incubations with oligomycin (2.4 μg/ml), a complex V inhibitor, enhanced the specific loss of COX activity mediated by azide (Table I), marked inhibition of ^{35}S incorporation into mitochondrial translation products (36.2% of control; data not shown) suggested that the oligomycin effect was attributable to reduced availability of mitochondrially encoded COX subunits required for holoenzyme assembly.

Chronic Azide Effects on COX Activity Are Independent of Changes in Bulk Phase ROS Production—Although other bioenergetic enzymes were unaffected by azide treatment, marked inhibition of CuZn SOD (12.0 ± 3.6% of control) and catalase (30.8 ± 1.3% of control) was also observed (Fig. 1*D*). This suggested the possibility that altered cellular capacity to metabolize ROS may contribute to the loss of COX activity. However, myoblasts treated for 24 h with 100 μM azide showed no increase in DCF fluorescence, an indicator of H_2O_2 production, despite 80% losses in COX activity (Figs. 2, *A* and *B*). Moreover, azide had no effect on catalase mRNA levels, a sensitive indicator of oxidative stress (data not shown).

To further confirm that azide effects on COX were not mediated by altered ROS production, C2C12 myotubes were treated for 24 h with a spectrum of pro-oxidants. Treatment with the O_2^- generator paraquat (45) and the catalase inhibitor aminotriazole (46) alone and in combination did not result in a loss of COX activity (Figs. 2, *C* and *D*). A complementary approach, in which myotubes were incubated with a series of antioxidants, also failed to prevent azide-induced COX losses. Treatments included supplementation with sodium selenite (47), which induced a 4-fold increase in glutathione peroxidase (*GPx*) content (Fig. 2*C*), and chemical antioxidants *N*-acetylcysteine (H_2O_2), Tiron (O_2^-), carnosine (O_2^-), and mannitol (hydroxyl radical) (each 0.1–1 mM) ($n = 3$, data not shown).

TABLE I

Azide and oligomycin effects on enzyme activity

C2C12 cells were differentiated for 6 days under serum-starved conditions and treated for 24 h with either 10 μM azide or 2.4 μg/ml oligomycin alone or in combination. COX, NADH ubiquinone oxidoreductase (NDH), catalase, and total SOD activities were assayed at 37 °C from solubilized cell extracts as outlined under "Experimental Procedures." All rates are expressed relative to controls ($n = 5$).

Treatment	Enzyme activity			
	COX	NDH	Catalase	Total SOD
		% control		
10 μM azide	53.2 ± 4.6[a]	112.3 ± 6.9	74.4 ± 4.8[a]	63.5 ± 4.8[a]
2.4 μg/ml oligomycin	91.5 ± 1.8	128.7 ± 4.2[a]	123.7 ± 4.9[a]	91.5 ± 8.2
10 μM azide + 2.4 μg/ml oligomycin	26.9 ± 5.1[a,b]	147.9 ± 20.4[a,b]	107.8 ± 7.3[b]	38.2 ± 3.8[a,b]

[a] Denotes a significant difference ($p < 0.05$) between control and treated cells.
[b] Denotes a significant difference ($p < 0.05$) between cells treated with azide alone versus azide plus oligomycin.

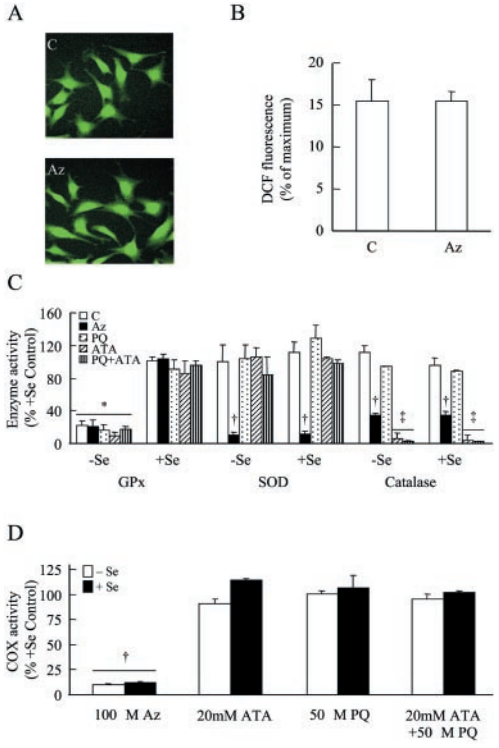

FIG. 2. **Azide effects on COX activity are not dependent on altered bulk phase ROS production.** *A*, DCF fluorescence in control (*C*) and azide-treated (*Az*, 100 μM, 24 h) myoblasts; *B*, *panel A*, expressed as a percentage of H_2O_2-stimulated maximal fluorescence ($n = 5$). *C*, activities of glutathione peroxidase (*GPx*), total cellular SOD, catalase; *D*, COX in day 6 serum-starved C2C12 myocytes grown either in minimal or sodium selenite (50 nM)-supplemented media and treated with azide (100 μM), aminotriazole (*ATA*, 20 mM), paraquat (*PQ*, 50 μM), or aminotriazole plus paraquat for 24 h (mean ± S.E., $n = 3$). All rates are expressed relative to sodium selenite-supplemented controls. Paraquat treatments in particular had pronounced effects on cellular ROS production, as indicated by elevated catalase mRNA levels (100% increase after 1 day) and increased total SOD and catalase activities (200 and 50% increase, respectively, after 3 days; data not shown). Chronic treatment with higher doses of paraquat resulted in loss of viability and cell death (data not shown). The *asterisk* denotes a significant difference ($p < 0.05$) between cells grown in minimal *versus* sodium selenite-supplemented media. The *single dagger* denotes a significant difference ($p < 0.05$) between azide-treated and control cells. The *double dagger* denotes a significant difference ($p < 0.05$) between azide- and ATA-treated cells.

NO is a well known, reversible inhibitor of COX (48, 49). One of its metabolic byproducts, $ONOO^-$, may also be catabolized by COX, a process that leads to irreversible catalytic inactivation of the enzyme (50). We therefore considered the potential contribution of altered NO and reactive NO species metabolism to the azide effects. No effects comparable with azide-dependent losses in COX were seen with the NO and $ONOO^-$ generators *S*-nitroso-*N*-acetylpenacillamine (SNAP) and 3-morpholinosydnonimine (SIN-1; 0.01–3 mM), the nitric-oxide synthase inhibitor *N*ω-nitro-L-arginine methyl ester hydrochloride (L-NAME; 0.1–1 mM), or the soluble guanylyl cyclase inhibitor 8-bromo-cGMP (5–500 nM) (data not shown).

Chronic Azide Treatment Does Not Affect the Levels of Mitochondrially and Nuclear-encoded COX mRNAs and Proteins—Northern and western analyses were conducted to evaluate whether the loss in COX activity was accompanied by changes in mRNA and/or protein levels of individual COX subunits. Levels of two mitochondrially encoded (COX I and II) and two nuclear-encoded subunit (COX IV and VIa) mRNAs were unchanged after 1, 3, or 6 days of treatment with 50 μM azide (Fig. 3*A*; $n = 5$). Lack of a general protection of COX II mRNA in response to azide-mediated COX inhibition (see Ref. 14) was further confirmed by measuring COX II/COX I mRNA ratios from azide titrations of ASMC, HEK293, and PC12 cells (data not shown). Protein levels of COX I, II, and IV subunits were also comparable in azide-treated and control cells after either 1 or 3 days of treatment with 100 μM azide (Fig. 3*B*). Similarly, levels of the ND1 subunit of complex I and of cytochrome *c*, the electron donor to complex IV, were unaffected by azide treatment.

To address whether azide inhibited the synthesis of individual subunits required for complex assembly without altering the levels of pre-existing ETC enzymes, the rate of mitochondrial protein synthesis was measured in control and azide-treated cells. Azide had no effect on the rate of mitochondrial protein synthesis, either acutely (added with label) or chronically (added 24 h before pulse-chase) (data not shown). Parallel treatment of myocytes with azide and thiamphenicol, a specific inhibitor of mitochondrial translation (51), revealed that both agents caused significant losses of COX activity without affecting the activities of complex I and CS (Fig. 4*A*). However, despite a 65% depression in the rate of mitochondrial protein synthesis relative to controls (Fig. 4*B*), the thiamphenicol-induced loss of COX activity was significantly slower than that observed with azide.

Chronic Azide Effects on Catalytic Activity Do Not Involve Copper Chelation—Irreversible losses of COX activity could also conceivably arise as a result of damage to either its metal centers or surrounding protein moieties. The ability of azide to act as a monodentate chelator of first transition series metals by binding to open coordination sites raised the possibility that it may exert its effects on COX activity by either chelation or stripping of copper centers within the holoenzyme. To evaluate the potential importance of ligand interactions with the copper centers in contributing to azide-induced COX losses, C2C12 cells were incubated with 10 μM azide in the presence or absence of copper ($CuCl_2$, Cu-His) and copper chelators (neocuproine, *BCS*) (Fig. 5*A*). Azide effects on COX activity were unperturbed when it was added in combination with a 10-fold molar excess of $CuCl_2$ or Cu-His. Treatment with either neocuproine or bathocuproine disulfonic acid failed to mimic the

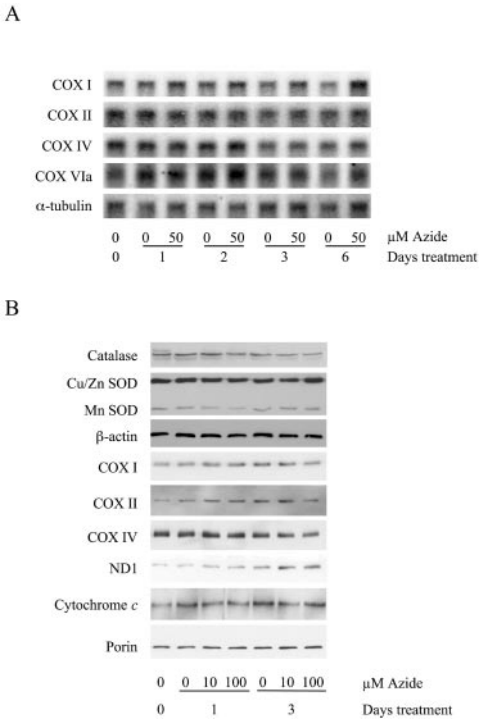

FIG. 3. **Azide effects on catalytic activity do not involve reduced steady-state mRNA or protein levels of individual COX subunits.** *A*, representative autoradiograms of COX I, II, IV, and VIa mRNA levels as a function of azide treatment (50 μM) over a 6-day period ($n = 5$). *B*, representative immunoblots for bioenergetic (COX I, II, and IV; ND1; cytochrome *c*) and ROS-scavenging (catalase, Cu/Zn-SOD and MnSOD) proteins as a function of treatment with azide (0, 10, 100 μM) for 3 days. Porin and actin were used as loading controls for bioenergetic and ROS-scavenging enzymes, respectively.

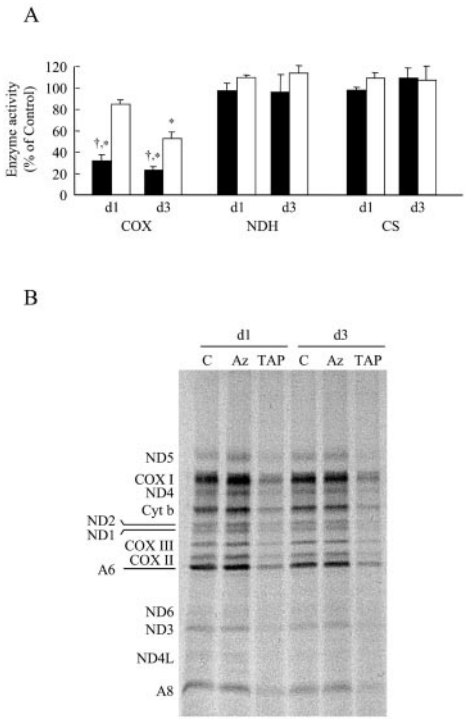

FIG. 4. **Azide effects on COX content are not mediated by inhibition of mitochondrial protein synthesis.** Day-6 serum-starved myotubes were treated with either 50 μM azide (*Az*, *solid bar*) or 50 μg/ml thiamphenicol (*TAP*; *open bar*) for 3 days. *C*, control. *A*, COX, NADH ubiquinone oxidoreductase (*NDH*), and CS activities were measured in solubilized cell extracts after 1 (*d1*) and 3 days (*d3*) of treatment. Rates are expressed relative to controls (mean ± S.E., $n = 3$). Higher thiamphenicol doses (up to 200 μg/ml) were ineffective at increasing the rate of loss of COX activity in TAP-treated cells (data not shown). The *asterisk* denotes a significant difference ($p < 0.05$) between treated and control cells. The *single dagger* denotes a significant difference ($p < 0.05$) between azide- and TAP-treated cells. *B*, ^{35}S pulse-labeling of mitochondrial translation products in control (*C*), azide and thiamphenicol (*TAP*)-treated myotubes after 1 (*d1*) and 3 (*d3*) days of treatment.

effects of azide on COX, although marginal losses in COX activity (~35% of total activity in 24 h) were observed. Moreover, the effects of azide and copper chelators on catalytic activity were additive, suggesting different modes of action.

Azide-mediated Loss of Catalytic Activity Is Accompanied by a Lesser Decline in COX Content, Which Reflects an Accelerated Rate of Holoenzyme Dissociation—In an attempt to further clarify the molecular basis of the defect in COX activity, difference spectra were generated from solubilized mitochondria. Although the levels of cytochromes *b* (268.2 *versus* 267.9 nM) and *c* (252.8 *versus* 274.7 nM) were comparable in control and azide-treated samples, cells exposed to 100 μM azide for 24 h displayed approximately a 65% reduction in cytochrome aa_3 content (40.3 *versus* 14.7 nM, $n = 2$; Fig. 5*B*). CO-derived difference spectra revealed that although cytochrome aa_3 content was markedly reduced in azide-treated cells, the integrity of the binuclear center of residual COX was preserved (data not shown). Subsequent analyses of total heme A content by reverse phase HPLC confirmed the loss of cytochrome aa_3 in azide-treated cells (heme A/protoheme −35.4 ± 6.7% of control, $n = 3$; Figs. 5, *C* and *D*).

Differential effects of azide on residual catalytic activity (15%; Fig. 2*D*) relative to cytochrome aa_3 content (35%; Fig. 5, *B–D*) prompted us to measure the temporal decline in each parameter over a refined inhibition time course. Significant, stepwise decreases in COX catalytic activity were observed in azide-treated cells at each time point over a 24-h period (Fig. 6, *A* and *C*). In contrast, COX content declined more slowly, with significant reductions initially observed after 6 h of azide treatment (Figs. 6, *A* and *C*). Pairwise comparisons confirmed that catalytic activity declined at a significantly greater rate relative to holoenzyme levels at all time points except for 2 h (Fig. 6*C*).

Despite significant temporal reductions in holoenzyme content, steady-state subunit levels of COX I and II were comparable between control and azide-treated cells at all time points (Fig. 7*A*). Immunoblots of second-dimension gels of 24 h control and azide-treated samples demonstrated that a disproportionate amount of the total pool of available COX I and II subunits in azide-treated cells were present as unassembled, full-length polypeptides (Fig. 7*B*). Collectively, these data argue that azide treatment leads to an initial loss of catalytic activity that ultimately results in an accelerated rate of holoenzyme dissociation, an event that may be triggered by irreversible damage to either the structure or coordination of its heme moieties.

DISCUSSION

The present study characterized the mechanism by which chronic azide treatment mediates irreversible losses in COX activity. Although azide has traditionally been used *in vitro* as a rapid and reversible inhibitor of COX activity (9, 10), it has been shown that chronic exposure results in "selective" and irreversible COX losses both *in situ* (14) and *in vivo* (15, 16). However, the mechanistic basis of these two distinct effects on COX had not been previously investigated. Because selective COX losses and Alzheimer's disease-like symptoms have been observed in rats chronically infused with azide (15, 52), this model may be clinically relevant to the pathophysiology of COX deficiency.

The primary finding of the present study is that chronic azide treatment causes the irreversible loss of COX activity by

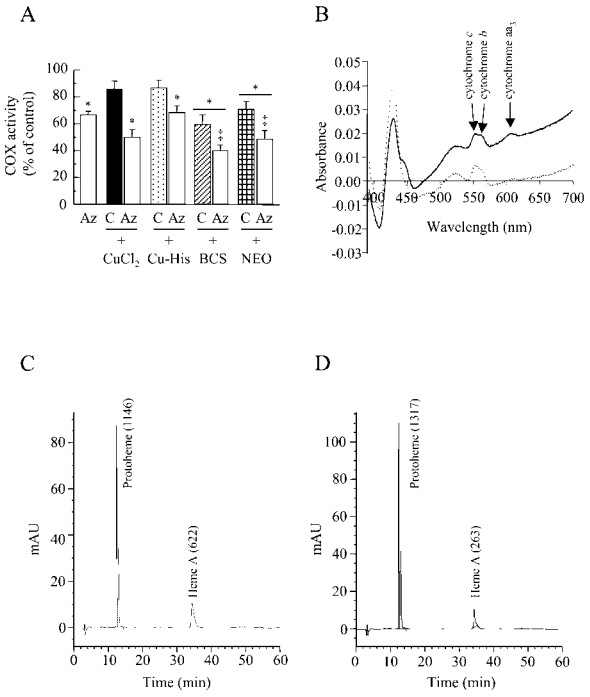

FIG. 5. **Azide-mediated loss of catalytic activity results in a lesser decline in aa_3 content that is independent of copper chelation.** *A*, day 6 serum-starved myotubes were treated for 24 h with azide (*Az*, 10 μM) in the presence or absence of 100 μM copper ($CuCl_2$, Cu-His) and copper chelators (neocuproine (*NEO*), bathocuproine disulfonic acid (*BCS*)). *C*, control. COX activity was measured in solubilized cell extracts, and rates were expressed relative to controls (mean ± S.E., $n = 3$). The *asterisk* denotes a significant difference ($p < 0.05$) between control and treated cells. The *double dagger* denotes a significant difference ($p < 0.05$) between cells treated with azide alone and azide plus copper chelators. *B–D*, day 6 serum-starved myotubes were treated with 0 or 100 μM azide for 24 h. *B*, difference spectra (air-oxidized minus dithionite-reduced) were generated from solubilized mitochondrial extracts to quantify cytochrome contents in both samples (control *versus* azide, $n = 2$: aa_3, 40.3 *versus* 14.7 nM; *b*, 268.2 *versus* 267.9 nM; *c*, 252.8 *versus* 274.7 nM). *C* and *D*, reverse phase HPLC profiles of protoheme (*first peak*) and heme A (*third peak*) content in control (*C*) and azide-treated (*D*) cells respectively ($n = 3$). The integrated area under the curve is shown in *parentheses*, with heme A content normalized to protoheme to account for different amounts of starting protein (control *versus* azide, $n = 3$: 0.498 ± 0.111 *versus* 0.167±.0.021).

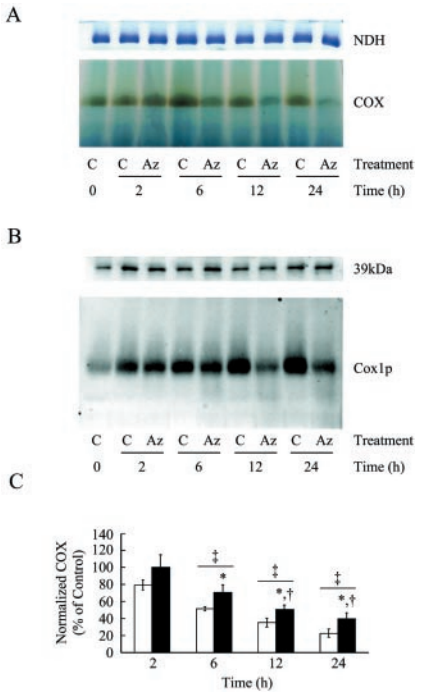

FIG. 6. **Azide-mediated losses in catalytic activity are more pronounced than declines in holoenzyme content.** Proliferating myoblasts were treated with 0 or 100 μM azide (*Az*) for 0, 6, 12, and 24 h. *C*, control. Mitoplasts were isolated from digitonin-permeabilized cells at a detergent to protein ratio of 1.2:1, solubilized (1.5 mg/ml) in lysis buffer (750 mM 6-aminocaproic acid, 50 mM Bistris (pH 7.0), and 1.5% laurylmaltoside) and electrophoresed under native conditions as described under "Experimental Procedures." *A*, in-gel assays (35) for COX and NADH ubiquinone oxidoreductase (*NDH*) activity after electrophoresis in the first dimension ($n = 3$). Complimentary measurements were also made spectrophotometrically using whole cell extracts ($n = 5$). *B*, first-dimension native gels were transferred using semi-dry immunoblotting (36) and incubated overnight at 4 °C with either anti-Cox1p (1:1,000) or anti-39-kDa (1:2,000) monoclonal antibodies (Molecular Probes). COX and NADH ubiquinone oxidoreductase holoenzyme levels were quantitated using an enhanced chemifluorescence detection system (Amersham Biosciences, Inc.) according to the manufacturer's specifications ($n = 3$). *C*, summary of azide-mediated declines in COX catalytic activity (*open bar*) and COX content (*solid bar*) over the course of a 24-h treatment. Significant ($p < 0.05$) stepwise reductions in COX catalytic activity were observed at each time point and are not presented on the graph for the sake of clarity. The *asterisk* denotes a significant difference ($p < 0.05$) in COX holoenzyme content between azide-treated cells relative to $t = 0$ control. The *single dagger* denotes a significant difference ($p < 0.05$) in COX holoenzyme content between azide-treated cells. The *double dagger* denotes a significant difference ($p < 0.05$) between residual COX catalytic activity and COX holoenzyme content in azide-treated cells at a given time point.

reducing holoenzyme content. Interestingly, the loss of COX activity was greater than the decline in COX content, as assessed by three independent indices (spectroscopy (aa_3), reverse phase HPLC (total heme A), and blue native PAGE (holoenzyme levels)). Such differential losses of COX activity and cytochrome aa_3 content also accompany COX deficiency in a number of disease models (Alzheimer's disease, Ref. 53; Menkes' syndrome, Ref. 54). The ability of azide to induce COX deficiency in the absence of any apparent defects in the expression of mitochondrially encoded and nuclear-encoded COX subunits represents another important finding and further emphasizes the potential shortcomings associated with using subunit profiles as a diagnostic tool to assess the molecular basis of COX deficiency (55).

Results from *in situ* and *in vitro* manipulations strongly suggest that chronic azide effects are not mediated by classic, high affinity interactions with the binuclear center (10). Although the K_i for this site is within the range of doses used in the present study (64 *versus* 0–100 μM), the binding of azide to the binuclear center is readily reversible *in vitro*. In contrast, extensive *in vitro* dialysis and *in vivo* washout of azide-treated cells failed to reverse *in situ* losses in COX activity. Co-incubation with cyanide was also unable to abrogate the observed azide effects despite its higher affinity for the binuclear center (40) and its ability to rapidly displace bound azide (41).

Our *in vitro* findings with purified COX, tissue homogenates, and cell culture extracts further extend the argument that azide-induced COX losses only occur *in situ*. The inhibition of COX activity in both muscle (C2C12, Sol 8, ASMC) and non-muscle (HEK293, PC12) cell backgrounds eliminates the possible requirement of either a muscle-specific factor or the expression of muscle-specific COX isoforms in order for azide to exert its effects. Although one possible *in vivo* influence common to all tissues is redox cycling of COX, inhibition of other OXPHOS complexes to alter the rate of electron flow to COX had no effects on the efficacy of azide. The minor losses in COX activity seen with oligomycin treatment in both control and azide-treated cells appear to arise through indirect inhibition of mitochondrial protein synthesis rather than to direct effects on COX.

COX has previously been shown to catalyze the one-electron

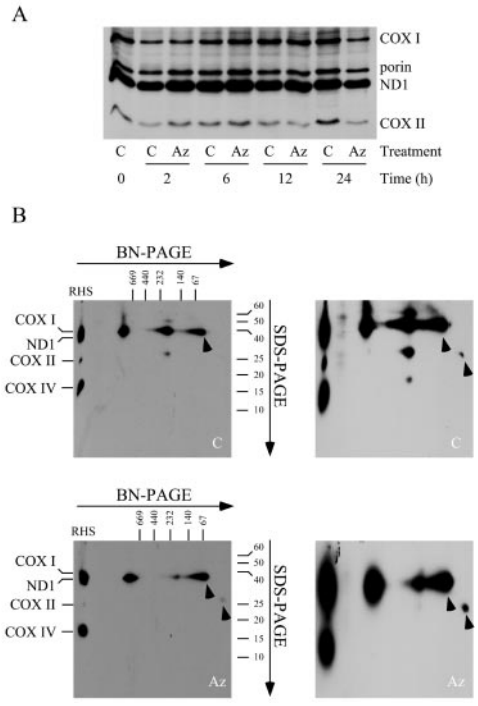

FIG. 7. **Azide-mediated losses in COX content reflect dissociation of the holoenzyme into its subunit aggregates.** Proliferating myoblasts were treated with 0 or 100 μM azide for 0, 6, 12, and 24 h. *A*, representative immunoblot for COX I, II, ND1, and porin steady-state protein levels in control (*C*) and azide (*Az*)-treated cells. Relevant information concerning antibody titers, conditions, and detection are described in detail under "Experimental Procedures." *B*, control and azide-treated lanes from first-dimension blue native (*BN*) gels (24-h time point) were denatured and run in the second dimension as previously described (36) alongside a denatured rat heart mitochondria standard (*RHS*) and a standard molecular weight marker (Invitrogen). After semi-dry immunoblotting of both gels onto the same membrane, membranes were incubated overnight at 4 °C with an antibody mixture containing COX I, COX II, COX IV, and ND1. Steady-state levels of all proteins were detected using standard luminol-enhanced chemiluminescence (Pierce) as described under "Experimental Procedures." The *arrows* in each *panel* designate unassembled COX I and II subunits. Longer exposures of otherwise identical images are represented on the right hand side of *panel B*.

oxidation of azide to the azidyl radical, a reaction that results in its irreversible inactivation *in vitro* (22). The analogous reaction with cyanide leads to loss of COX activity by cyanyl radical attack of the non-metal protein matrix intrinsic to the holoenzyme (57). Although azidyl radical attack could in principle lead to COX damage and dissociation in our model, this seems improbable for at least two reasons. First, *in vitro* catalysis of azide to the azidyl radical requires very high levels of exogenous H_2O_2 (1 mM) and azide (10 mM). In contrast, manipulation of cellular capacity either to produce (*i.e.* aminotriazole-mediated catalase inhibition, ETC inhibitors) or scavenge (*i.e. N*-acetylcysteine, sodium selenite-induced increases in glutathione peroxidase content) H_2O_2 did not alter the azide effects on COX. Second, unlike cyanyl-inhibited COX, which showed no alteration in difference spectra (57), spectroscopically detectable cytochrome aa_3 content was reduced as a result of azide treatment.

Several lines of evidence also argue against the involvement of bulk phase ROS production in azide-mediated COX losses despite the additional inhibition of catalase and CuZn SOD activities. First, extensive experimental manipulations that resulted in significant changes in the ability to either produce or quench a suite of free radicals (H_2O_2, hydroxyl radical, NO, $ONOO^-$, O_2^-) had no effect on COX activity. Second, inhibition of complexes I and III, the two major intramitochondrial sites of O_2^- production (58), neither contributed to nor abrogated azide-mediated losses of COX activity. And third, the activity of aconitase, a mitochondrial hemoprotein that is highly sensitive to oxidative stress (59, 60), was the same in control and azide-treated cells (data not shown).

Reduced COX content does not appear to be due to its impaired synthesis since azide had no effect on levels of COX mRNA transcripts (I, II, IV, VIa), COX subunits (I, II, IV), mtDNA copy number (14), or the rate of mitochondrial protein synthesis. Although potential azide effects on the expression of the 10 remaining subunits were not assessed, the azide phenotype differs dramatically from those observed in model systems in which either the proteolytic or chaperone function of mitochondrial proteases has been modified (61, 62) or in which the function of nuclear-encoded proteins critical to complex assembly has been lost (5, 64). Therefore it seems unlikely that defects in COX biogenesis contributed to the azide-induced loss of holoenzyme content. Lack of an equivalent temporal loss of COX activity in thiamphenicol-treated cells provides further support that azide-mediated COX losses occur at a much greater rate than can be accounted for simply by the kinetic inhibition of processes relating to complex biogenesis.

The inability of either copper to abrogate or copper chelators to mimic the azide effects further suggests that the loss of catalytic activity was independent of azide interactions with the copper centers within COX. Although previous *in vitro* studies have described alterations in the kinetic and spectroscopic properties of COX in response to incubation with bathocuproine disulfonic acid, substantially higher doses were required to obtain an effect (0.05–1 M; Refs. 56 and 63). In contrast, comprehensive spectroscopic, HPLC, and native electrophoretic analyses all argue that structural damage either at or near the heme groups caused an initial loss of catalytic activity that ultimately led to a reduction in COX content through holoenzyme dissociation. The ability of azide to mediate the differential loss of catalytic activity and holoenzyme content and to induce COX deficiency without altering the expression of mitochondrially encoded and nuclear-encoded COX subunits has important implications for diagnostic tools used to assess the molecular basis of COX deficiency.

Acknowledgments—We gratefully acknowledge the continued technical support received from Dr. E. A. Shoubridge and his lab (Montreal Neurological Institute, Canada).

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