Modulation of Mitochondrial Complex I Activity by Reversible Ca^{2+} and NADH Mediated Superoxide Anion Dependent Inhibition†

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ABSTRACT: Complex I, a key component of the mitochondrial respiratory chain, exhibits diminished activity as a result of cardiac ischemia/reperfusion. Cardiac ischemia/reperfusion is associated with increases in the levels of mitochondrial Ca^{2+} and pro-oxidants. In the current in vitro study, we sought evidence for a mechanistic link between Ca^{2+}, pro-oxidants, and inhibition of complex I utilizing mitochondria isolated from rat heart. Our results indicate that addition of Ca^{2+} to solubilized mitochondria results in loss in complex I activity. Ca^{2+} induced a maximum decrease in complex I activity of approximately 35% at low micromolar concentrations over a narrow physiologically relevant pH range. Loss in activity required reducing equivalents in the form of NADH and was not reversed upon addition of EGTA. The antioxidants *N*-acetylcysteine and superoxide dismutase, but not catalase, prevented inhibition, indicating the involvement of superoxide anion ($O_2^{\bullet -}$) in the inactivation process. Importantly, the sulfhydryl reducing agent DTT was capable of fully restoring complex I activity implicating the formation of sulfenic acid and/or disulfide derivatives of cysteine in the inactivation process. Finally, complex I can reactivate endogenously upon Ca^{2+} removal if NADH is present and the enzyme is allowed to turnover catalytically. Thus, the present study provides a mechanistic link between three alterations known to occur during cardiac ischemia/reperfusion, mitochondrial Ca^{2+} accumulation, free radical production, and complex I inhibition. The reversibility of these processes suggests redox regulation of Ca^{2+} handling.

The respiratory chain functions to transfer electrons from intracellular reducing equivalents to molecular oxygen, thereby enabling the translocation of protons across the inner mitochondrial membrane and the establishment of an electrochemical gradient required for the production of ATP. Complex I, a component of the mitochondrial respiratory chain, catalyzes the first step in the utilization of electrons from NADH. As such, complex I would be expected to play a pivotal role in the maintenance of the proton gradient and energy production. Deficits in the activity of complex I have been observed in animal models of cardiac ischemia/reperfusion (*1*−*5*). Loss in activity would be expected to exert significant effects on mitochondrial and cellular function. Nevertheless, molecular event(s) responsible for declines in complex I activity and the cellular and physiological consequences of these occurrences have not been fully delineated. Consideration of the intracellular environment that exists during cardiac ischemia/reperfusion would provide insight into potential events responsible for alterations in complex I activity.

Depletion of tissue oxygen during ischemia results in declines in the rate of oxidative phosphorylation, depletion of creatine phosphate and ATP, and cellular acidosis (*1*−*10*). Decreases in both ATP concentration and pH adversely affect the ability of cells and organelles to maintain ionic gradients, thereby promoting cellular and mitochondrial Ca^{2+} overload (*10*−*12*). Reperfusion of ischemic cardiac tissue results in an even greater increase in mitochondrial Ca^{2+} concentration (*10*−*12*). Cardiac mitochondria can act as cellular buffers for Ca^{2+} (*13*). Mitochondrial import of Ca^{2+} is a dynamic process depending to a great extent on the proton gradient established by the electron transport chain (*13*−*16*). Inhibition of the electron transport chain or uncoupling of mitochondrial respiration from oxidative phosphorylation, processes that lead to a drop in the proton gradient across the mitochondrial membrane, have been shown to result in a marked decline in mitochondrial Ca^{2+} uptake (*17*−*19*). It is therefore of interest to assess the effects of Ca^{2+} on specific electron transport chain components and determine how these processes are affected by alterations in intracellular pH.

An additional facet of cardiac ischemia/reperfusion of potential importance in complex I inhibition is the production of superoxide anion ($O_2^{\bullet -}$) and pro-oxidants (*20*−*22*). Mitochondria play a key role in this process, exhibiting enhanced rates of free radical production upon reperfusion of ischemic myocardial tissue (*11*, *23*−*27*). It has previously been shown that, upon exposure of respiring mitochondria or submitochondrial particles to pharmacological inhibitors of complex I, mitochondria exhibit marked increases in the production of oxygen-derived free radicals (*28*−*30*). Free radicals are known to be highly reactive species capable of reacting with and modifying proteins, thereby leading to

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alteration in their activity (*31*). Importantly, exposure of isolated mitochondria and submitochondrial particles to oxygen radicals and pro-oxidants results in the inactivation of complex I (*32*−*38*). Thus, in addition to acidosis and Ca^{2+} overload, the production of free radical species must be investigated as a factor likely to contribute to loss in complex I activity during cardiac ischemia/reperfusion.

In the present study, the effects of pH, Ca^{2+}, and endogenously produced oxygen radicals on complex I activity were explored in an effort to provide insight into potential mechanisms responsible for loss in complex I activity during cardiac ischemia/reperfusion. Solubilized mitochondria were utilized to study the effects on complex I activity directly. Evidence is provided that complex I is inhibited by Ca^{2+} at low micromolar concentrations and at physiologically relevant pH values. The mechanism of inhibition involves Ca^{2+}-mediated NADH-dependent oxidative modification. Importantly, complex I was inhibited by free radicals produced endogenously and reactivated upon chelation of Ca^{2+} in the presence of the enzyme substrate, NADH. These findings are discussed in light of the potential role these processes play in regulation of calcium homeostasis.

MATERIALS AND METHODS

Reagents and Animals. Antimycin A, ubiquinone-1, superoxide dismutase (SOD)[1], catalase, thioredoxin, thioredoxin reductase, and glutaredoxin were purchased from Sigma. Male Sprague−Dawley rats (250−300 g) were obtained from Zivic Miller Laboratories.

Mitochondrial Isolation. Subsarcolemmal mitochondria were isolated from hearts of male Sprague−Dawley rats. Animals were anesthetized by intraperitoneal injection of urethane (2.4 g/kg) followed by thoracotomy. Hearts (0.9−1.1 g) were then removed and immediately immersed and rinsed in ice cold homogenization buffer containing 180 mM KCl, 5.0 mM MOPS, and 2.0 mM EDTA, pH 7.4. Tissue was minced and homogenized in 20 mL of homogenization buffer for ~5.0 s using a Polytron P10−35 homogenizer with setting adjusted to 3. The homogenate was centrifuged at 500*g* for 5.0 min (5 °C), and the resulting supernatant was filtered through cheesecloth. The mitochondrial pellet was obtained upon centrifugation of the supernatant at 5000*g* for 10 min (5 °C). After two rinses with 10 mM MOPS, pH 7.4, the mitochondria were resuspended in 10 mM MOPS, pH 7.4 to a final protein concentration of 5.0 mg/mL. Mitochondrial preparations were stored at −70 °C for later use. Protein determinations were performed using the bicinchoninic acid method (Pierce), with bovine serum albumin as a standard. Mitochondria prepared in this manner do not exhibit significant contamination of other sub-cellular or membrane components as judged by electron microscopic analysis (39).

Complex I Assay. Rat heart mitochondria at 5.0 mg/mL in hypotonic solution (10 mM MOPS, pH 7.4) were solubilized by two cycles of free-thawing using liquid nitrogen. Frozen and thawed mitochondria were then diluted to 5.0 μg/mL in a solution containing 10 mM MOPS, 10 nM antimycin A, 20 μM KCN, and 40 μM ubiquinone-1.

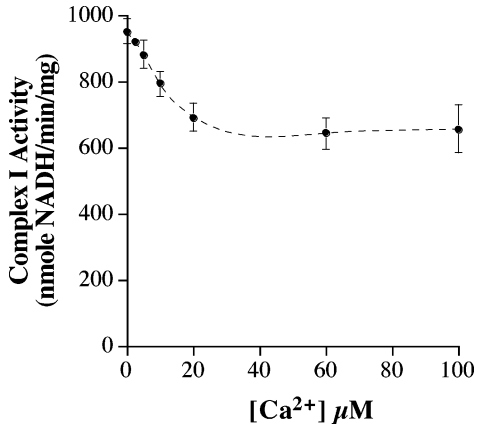

FIGURE 1: Ca^{2+}-induced complex I inhibition. Mitochondria isolated from rat heart were diluted to a protein concentration of 1.0 mg/mL in hypotonic solution (10 mM MOPS, pH 7.4) and solubilized by freeze−thawing as described in Materials and Methods. Mitochondrial protein was then diluted to 5.0 μg/mL in a solution containing 10 mM MOPS, pH 7.4, 10 nM antimycin A, 20 μM KCN, and 40 μM ubiquinone-1. Ca^{2+} was included in the assay mixture at concentrations indicated on the *abscissa*. NADH (40 μM) was then immediately added to initiate enzyme catalysis. Complex I activity was monitored spectrophotometrically as the oxidation of NADH at 25 °C. Each data point represents the mean and standard deviation of five separate experiments.

The pH of this solution was adjusted based on the experiment being conducted, as indicated in Results. NADH (5−40 μM) was then immediately added to initiate complex I activity. Complex I activity was measured as the rate of NADH oxidation (340 nm, $\epsilon = 6200\ M^{-1} \cdot cm^{-1}$) using a diode array UV spectrophotometer (Agilent). Measurable activity required the presence of ubiquinone-1 and was completely inhibited by rotenone indicating that NADH utilization was dependent on complex I activity.

Ca^{2+}, NADH, and Ca^{2+}/NADH Incubations. Complex I activity was measured after exposure of frozen and thawed mitochondria to various conditions. For experiments presented in Figures 1−3, Ca^{2+} was added to the assay mixture just prior to addition of NADH and initiation of complex I activity at concentrations indicated. In the experiments depicted in Figures 4−7, frozen and thawed mitochondria were pretreated with 2.5 μM NADH, 5.0 μM Ca^{2+}, or 5.0 μM Ca^{2+}, and 2.5 μM NADH for 30 s at 25 °C followed by addition of the 10 nM antimycin A and 20 μM KCN. Complex I activity was then initiated by addition of NADH (40 μM) and ubiquinone-1 (20 μM).

Pre- and Post-treatment with EGTA, N-Acetylcysteine (NAC), SOD, Catalase, DTT, and/or NADH. To determine whether EGTA, NAC, SOD and/or catalase prevents Ca^{2+}/NADH induced complex I inhibition, 8.0 μM EGTA, 4.0 mM NAC, 100 units/mL SOD, or 100 units/mL catalase were added to the preincubation mixture prior to addition of frozen and thawed mitochondria. The potential for reversal of complex I inhibition was tested by adding EGTA (100 μM), NAC (4.0 mM), SOD (100 units/mL), NADH (5.0 μM) and/or DTT (2.0 μM) after preincubation with Ca^{2+}/NADH (5.0 μM/2.5 μM), 30 s prior to addition of 10 nM antimycin A, 20 μM KCN, 40 μM ubiquinone-1, and NADH (40 μM) to initiate measurement of enzyme activity.

Treatment with Enzymatic Systems for Reduction of Protein Disulfides and Mixed Disulfides. The potential for reversal of complex I inhibition by enzymatic systems was

[1] ABBREVIATIONS: SOD, superoxide dismutase; NAC, *N*-acetylcysteine; Trx, thioredoxin; TrxR, thioredoxin reductase; GRx, glutaredoxin; GSH, reduced glutathione.

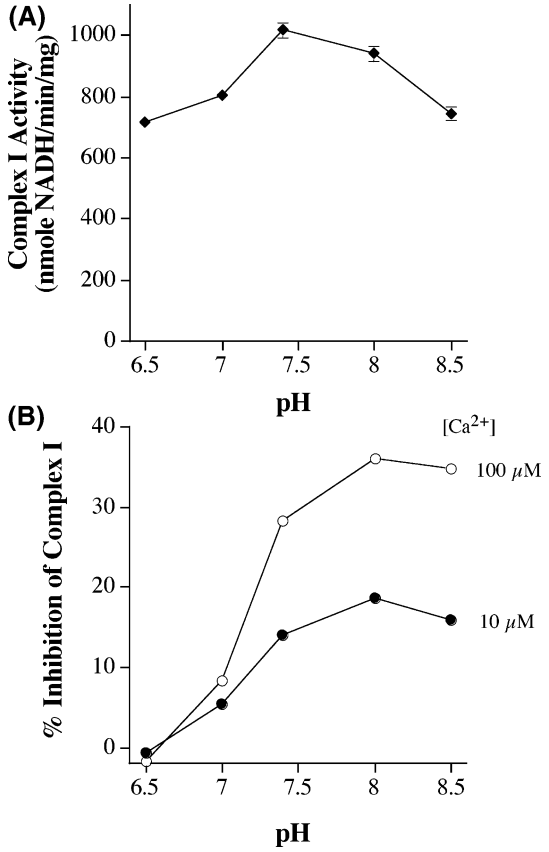

FIGURE 2: Effect of pH on complex I activity and Ca^{2+}-induced inhibition. Frozen and thawed mitochondria (5.0 μg protein/ml) were evaluated for complex I activity in the absence (♦) or presence of 10 (●) or 100 μM (○) Ca^{2+} at pH values ranging from 6.5 to 8.5 as indicated on the *abscissa* (Materials and Methods). Complex I assay conditions were as described in the legend to Figure 1. (A) Complex I activity was measured at pH 6.5−8.5 in the absence of Ca^{2+}. (B) Percentage loss in complex I activity in the presence of Ca^{2+} relative to values obtained in the absence of Ca^{2+} at corresponding pH values. Each data point represents the mean and standard deviation of five separate experiments.

tested by adding thioredoxin/thioredoxin reductase/NADPH (5.0 μM/5.0 μM/10 μM) or glutaredoxin/GSH (1.0 μM/0.5 mM) after preincubation with Ca^{2+}/NADH (5.0 μM/2.5 μM), 30 s prior to addition of 10 nM antimycin A, 20 μM KCN, 40 μM ubiquinone-1, and NADH (40 μM) to initiate measurement of enzyme activity.

RESULTS

Effect of Ca^{2+} on Complex I Activity. Exposure of solubilized mitochondria to micromolar concentrations of Ca^{2+} resulted in partial inhibition of complex I. The effects of Ca^{2+} were assessed utilizing frozen and thawed mitochondria isolated from rat heart. Prior to addition of NADH and initiation of enzymatic activity, Ca^{2+} was added at concentrations ranging from 0 to 100 μM. As shown in Figure 1, this resulted in a concentration dependent decrease in complex I activity. Near maximum inhibition was achieved at 20 μM Ca^{2+}, with an IC_{50} of approximately 10 μM under the conditions of our experiments. Ca^{2+} induced a maximum decrease in complex I activity of approximately 35%. Increasing Ca^{2+} concentrations up to 1.0 mM failed to augment the magnitude of inhibition. The inability to induce full inactivation of complex I may reflect several possibilities: (1) the enyzme may exist in different conformations and/or states which are differentially sensitive to Ca^{2+} (40−43); (2) Ca^{2+} may serve to regulate activity by affecting the kinetic parameters of the enzyme; and/or (3) the flow of electrons through complex I may be affected by Ca^{2+} and/or alternative endogenous electron acceptors may exist which preferentially interact with the modified enzyme. Importantly, ubiquinone-1, a standard artificial electron acceptor utilized for measurement of complex I activity, may interact at multiple sites on the enzyme (*44*, *45*). Thus, assays for complex I using ubiquinone-1 may underestimate the magnitude of enzyme inhibition. This is exemplified by the fact that when the electron acceptor ferricyanide was utilized to measure complex I activity, no enzyme inhibition was observed (not shown). This result also indicates that complex I is inhibited at a site(s) distal to where ferricyanide accepts electrons, close to the site of NADH oxidation (*42*, *46*).

Effect of pH on Ca^{2+}-Induced Inhibition of Complex I. During cardiac ischemia, Ca^{2+} overload is preceded by acidosis. The rate and extent to which normal pH is re-established during reperfusion is dependent on the duration of ischemia (*10*, *12*, *47*). It was therefore important to determine whether the sensitivity of complex I to Ca^{2+}-induced inhibition is affected by variations in pH. For this set of experiments, complex I was assayed in frozen and thawed mitochondria in the presence of 0, 10, or 100 μM Ca^{2+} at pH values ranging from 6.5 to 8.5. In the absence of Ca^{2+}, optimal activity was observed at pH 7.4, with a relative decline of 25% when pH was lowered to 6.5 or raised to 8.5 (Figure 2A). In the presence of Ca^{2+}, complex I activity was unaffected at pH 6.5 with maximum inhibition observed at pH 8.0 (Figure 2B). At both 10 and 100 μM Ca^{2+}, half-maximal inhibition was observed at approximately pH 7.2, with relatively little increase in the degree of inactivation above pH 7.4 (Figure 2B). Thus, complex I is responsive to Ca^{2+} over a narrow physiological pH range. In addition, increasing the Ca^{2+} concentration (10−100 μM) appears to affect the level of inhibition and not the pH where complex I becomes responsive to Ca^{2+} (Figure 2B).

Kinetics Analysis of Complex I Inhibition by Ca^{2+}. To gain insight into the mechanism by which Ca^{2+} inhibits complex I, kinetic analyses were performed. Frozen and thawed mitochondria were assayed for complex I activity with varying concentrations of NADH (5−40 μM) in the absence and presence of 20 μM Ca^{2+}. As shown in Figure 3A, the relative level of Ca^{2+}-induced complex I inhibition was enhanced with increasing NADH concentrations. Addition of Ca^{2+} resulted in a decline in the maximum rate (V_{max}) of the enzyme from 1560 ± 42 to 979 ± 34 nmole NADH/min/mg ($P \leq 0.0001$) (Figure 3B). Furthermore, the K_M of the enzyme for NADH decreased from 11.4 to 5.7 μM in the presence of Ca^{2+} ($P \leq 0.0001$) (Figure 3B). Thus, NADH promotes Ca^{2+}-induced inhibition of complex I, suggesting that the effect of Ca^{2+} on enzyme activity is mediated by NADH. The possibility that formation of an NADH−Ca^{2+} complex (48) either diminishes the concentration of substrate necessary for catalysis or binds to the active site but is turned over at a slower rate can be excluded. For each of these mechanisms, competitive inhibition would be observed. It is therefore likely that Ca^{2+}-induced inhibition of complex I requires enzyme turnover and thus only occurs in the presence of the enzyme's substrate NADH.

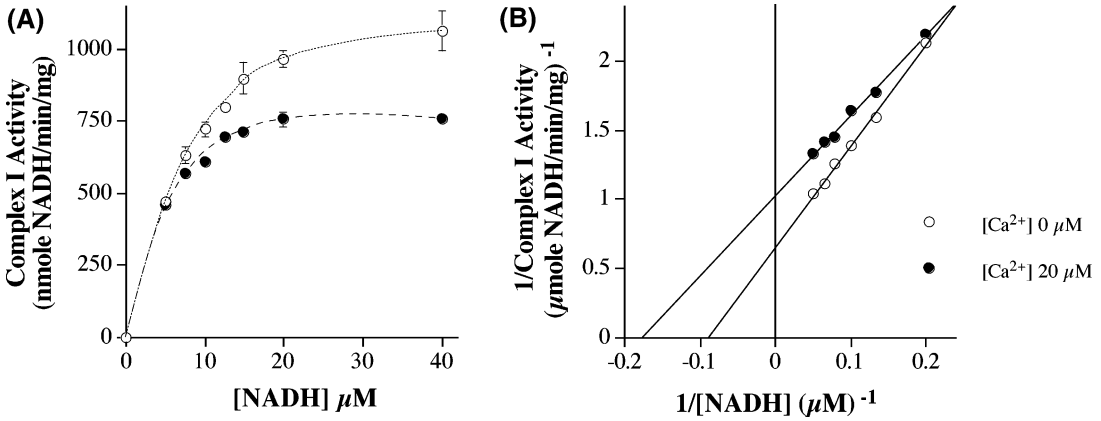

FIGURE 3: Effect of NADH concentration on Ca^{2+}-induced complex I inhibition. Frozen and thawed mitochondria (5.0 μg protein/ml) were assayed for complex I activity in a solution containing 10 mM MOPS, pH 7.4, 10 nM antimycin A, 20 μM KCN, and 40 μM ubiquinone-1. NADH was added at concentrations indicated on the *abscissa* to initiate complex I activity. (A) Assays were performed under initial rate conditions in the absence (○) or presence (●) of 20 μM Ca^{2+}. (B) Double reciprocal plot of rate vs [NADH] (Lineweaver−Burk plot). Each data point represents the mean and standard deviation of three separate experiments.

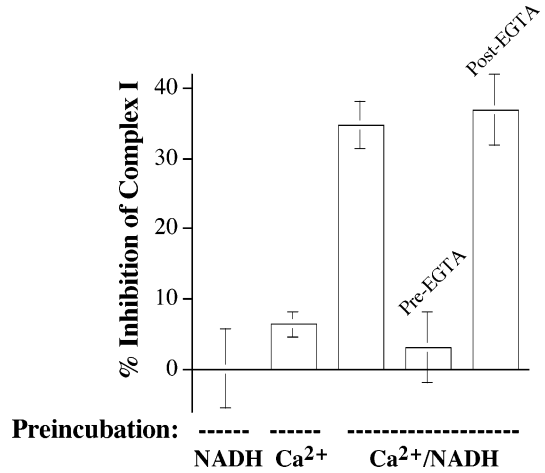

FIGURE 4: Factors required for complex I inhibition. Frozen and thawed mitochondria (5.0 μg protein/ml) were incubated with 2.5 μM NADH, 5.0 μM Ca^{2+}, or 5.0 μM Ca^{2+} and 2.5 μM NADH as indicated on the *abscissa* for 30 s in 10 mM MOPS, pH 7.4 at 25 °C. This was followed by addition of 10 nM antimycin A, 20 μM KCN, 20 μM ubiquinone-1, and 40 μM NADH to initiate complex I activity. Where indicated, EGTA (8.0 μM) was included in the preincubation mixture (pre-EDTA) or EGTA (100 μM) was added to the assay mixture after preincubation (post-EGTA). Each data point represents the mean and standard deviation of five separate experiments.

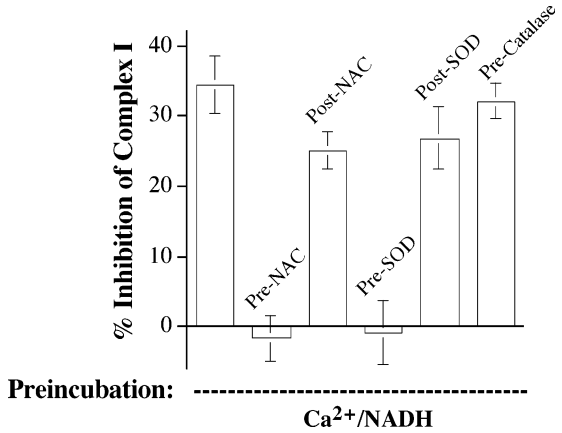

FIGURE 5: Role of superoxide anion ($O_2^{\bullet -}$) in Ca^{2+}-induced complex I inhibition. Frozen and thawed mitochondria (5.0 μg protein/ml) were preincubated with 5.0 μM Ca^{2+} and 2.5 μM NADH for 30 s in 10 mM MOPS, pH 7.4 at 25 °C. This was followed by addition of 10 nM antimycin A, 20 μM KCN, 20 μM ubiquinone-1, and 40 μM NADH to initiate complex I activity. Where indicated, NAC (4.0 mM), SOD (100 units/mL), or catalase (100 units/mL) was included in the preincubation mixture (pre-NAC, pre-SOD, or pre-catalase) or was added to the assay mixture after preincubation (post-NAC or post-SOD). Each data point represents the mean and standard deviation of five separate experiments.

Effect of Preincubation of Mitochondria with NADH, Ca^{2+}, or Ca^{2+}/NADH. Further characterization of the mechanism responsible for Ca^{2+}-induced NADH-dependent complex I inhibition was accomplished by preincubating frozen and thawed mitochondria with either NADH (2.5 μM), Ca^{2+} (5.0 μM), or Ca^{2+}/NADH (5.0/2.5 μM) for 30 s. NADH (40 μM), ubiquinone-1 (20 μM), antimycin A (10 nM), and KCN (20 μM) were then added at concentrations required for the specific measurement of the maximum activity of complex I. As shown in Figure 4, complex I was not inhibited when mitochondria were preincubated with NADH. A small decrease in activity (5%), similar to that seen when 5.0 μM Ca^{2+} was included in the assay mixture (Figure 1), was observed upon preincubation with Ca^{2+} (Figure 4). Importantly, the presence of both Ca^{2+} and NADH induced a marked decline in complex I activity (34%). Extension of the preincubation period to 1.0 min did not increase the magnitude of inhibition observed in the presence of Ca^{2+}/NADH (not shown). When solubilized mitochondria were preincubated with Ca^{2+}/NADH in the presence of the complex III inhibitor, antimycin A, and the complex IV inhibitor, KCN no inhibition of complex I was observed indicating the requirement for enzyme turnover (not shown). Addition of EGTA prior to Ca^{2+}/NADH prevented inhibition (Figure 4). In striking contrast, EGTA added after the preincubation period failed to reverse the Ca^{2+}/NADH induced inhibition (Figure 4). These findings suggest the occurrence of a conformational change to complex I that renders Ca^{2+} inaccessible to EGTA and/or an alternative form of modification induced by Ca^{2+}/NADH. It is interesting to note that preincubation with 5.0 μM Ca^{2+} and 2.5 μM NADH was found to induce maximum inhibition of complex I (~35%). This is in contrast to the relatively low degree of inhibition (~7.0%) observed when 5.0 μM Ca^{2+} was added directly to the assay mixture (Figure 1). The low level of

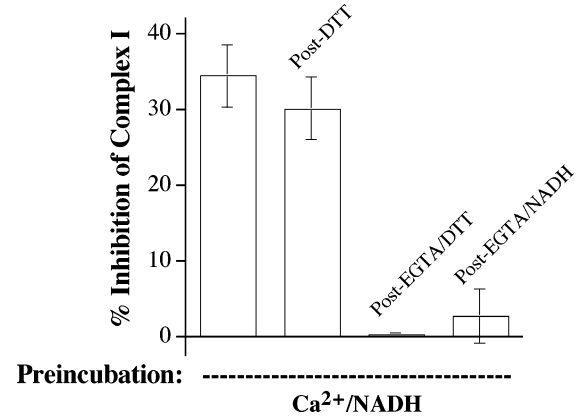

FIGURE 6: Reversal of complex I inhibition by the sulfhydryl reducing agent, DTT or NADH. Frozen and thawed mitochondria (5.0 μg protein/ml) were preincubated with 5.0 μM Ca^{2+} and 2.5 μM NADH for 30 s in 10 mM MOPS, pH 7.4 at 25 °C. This was followed by addition of 10 nM antimycin A, 20 μM KCN, 20 μM ubiquinone-1, and 40 μM NADH to initiate complex I activity. Where indicated, DTT (2.0 μM), DTT (2.0 μM) in combination with EGTA (100 μM), or NADH (5.0 μM) in combination with EGTA (100 μM) was added after preincubation, 30 s prior to measurement of complex I activity. Each data point represents the mean and standard deviation of five separate experiments.

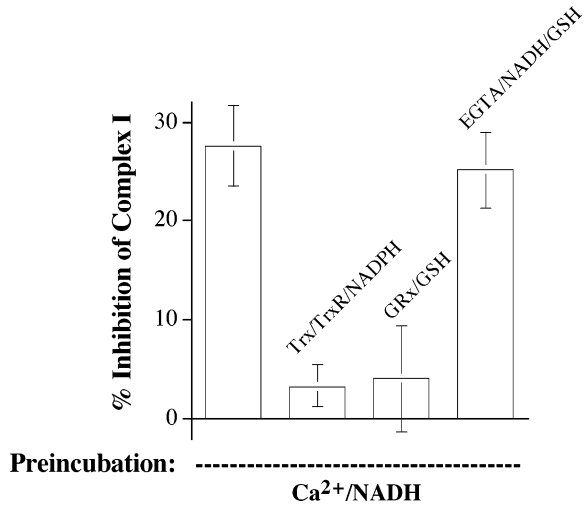

FIGURE 7: Reversal of complex I inhibition by enzymatic protein disulfide and mixed disulfide reducing systems. Frozen and thawed mitochondria (5.0 μg protein/mL) were preincubated with 5.0 μM Ca^{2+} and 2.5 μM NADH for 30 s in 10 mM MOPS, pH 7.4 at 25 °C. This was followed by addition of 10 nM antimycin A, 20 μM KCN, 20 μM ubiquinone-1, and 40 μM NADH to initiate complex I activity. Where indicated, thioredoxin/thioredoxin reductase/NADPH (5.0 μM/5.0 μM/10 μM) or glutaredoxin/GSH (1.0 μM/0.5 mM) was added after preincubation, 30 s prior to measurement of complex I activity. Alternatively, GSH (0.5 mM) was added after preincubation. This was followed 30 s later by the addition of NADH (5.0 μM) in combination with EGTA (100 μM). Complex I activity was measured 30 s after addition of NADH/EGTA. Each data point represents the mean and standard deviation of five separate experiments.

inhibition observed in the absence of preincubation was found to be due to the presence of exogenously added ubiquinone-1, necessary for evaluation of enzyme activity (not shown). Ubiquinone-1 readily accepts electrons from complex I (*49*). These results therefore provide support for the involvement of reducing equivalents in complex I inhibition.

Effects of Free Radical Scavengers on Ca^{2+}-Induced Complex I Inhibition. Complex I is known to be a source of (*50*, *51*). We therefore explored the possibility that free radical events mediate Ca^{2+}-induced inhibition of complex I. As shown in Figure 5, the presence of the free radical scavenger NAC during preincubation with Ca^{2+}/NADH completely prevented complex I inhibition. Addition of NAC after preincubation with Ca^{2+}/NADH failed to significantly reverse inhibition. These results indicate that a free radical species is involved in modification and inhibition of complex I and provide a plausible explanation for the requirement for NADH in Ca^{2+}-induced inhibition. NADH would be a source of electrons necessary for free radical production. To gain further evidence for free radical involvement and to identify the species responsible for Ca^{2+}-induced NADH-dependent inhibition of complex I, the $O_2^{\bullet-}$ scavenger SOD was utilized. Preincubation of cardiac mitochondria with Ca^{2+}/NADH in the presence of SOD resulted in complete prevention of Ca^{2+}-induced complex I inhibition (Figure 5). In contrast, the use of heat denatured SOD failed to prevent complex I inhibition indicating the requirement for enzyme activity (not shown). Commercial sources of SOD often contain catalase as a contaminant. To rule out the potential involvement of H_2O_2 in complex I inactivation, catalase was included during preincubation with Ca^{2+}/NADH. As shown in Figure 5, catalase failed to prevent complex I inhibition, indicating that H_2O_2 is not involved in enzyme inhibition. Similar to results obtained with NAC, addition of SOD after preincubation with Ca^{2+}/NADH failed to reverse inhibition (Figure 5). These results strongly indicate that interaction of Ca^{2+}/NADH with complex I induces $O_2^{\bullet-}$-dependent oxidative modification and inhibition of the enzyme. Preparation of sub-mitochondrial particles is often utilized to remove endogenous antioxidant systems such as SOD and catalase. The maximum degree of Ca^{2+}/NADH-induced complex I inhibition (~35%) observed for disrupted mitochondria was not altered when sub-mitochondrial particles were utilized. Therefore, it does not appear that the presence of SOD at low concentrations in dilute, disrupted (frozen and thawed) mitochondria limits the level of complex I inhibition observed (Figure 5).

Chemical Characterization of Complex I Inhibition. Experiments were undertaken to provide chemical evidence for a role of free radicals in Ca^{2+}/NADH-mediated complex I inhibition and to identify likely forms of oxidative modification responsible for this process. Oxygen radicals have the potential to modify various amino acid side chains. Of these, the sulfur atom of cysteine is perhaps the most susceptible to free radical modification. Cysteine can be oxidized to sulfenic, sulfinic, and sulfonic acid (*52*, *53*). In addition, oxidation can lead to the formation of a disulfide bond between two cysteine residues (*54*, *55*). The sulfhydryl reducing agent DTT is capable of reducing disulfide bonds and sulfenic acid but cannot act on higher oxidations states of sulfur, such as sulfinic and sulfonic acid derivatives. As shown in Figure 6, addition of DTT to frozen and thawed mitochondria after preincubation with Ca^{2+}/NADH failed to reverse the observed inhibition of complex I. However, when DTT was added in combination with EGTA, enzyme activity was completely restored (Figure 6). DTT can interfere with the complex I assay by reducing ubiquinone-1. For this

reason, DTT was used at a low concentration (2.0 μM) in these experiments. It is therefore likely that DTT reverses oxidative modification to a sulfhydryl residue(s) responsible for inhibition of enzyme activity. The requirement for EGTA indicates that Ca^{2+} must be chelated to prevent continued Ca^{2+}-inducd $O_2^{\bullet-}$ production and enzyme inactivation upon consumption of low levels of DTT. NAC or SOD in combination with EGTA were unable to reverse complex I inhibition. Thus, oxidative modification of protein cysteine(s) appears to be the terminal event in enzyme inhibition requiring a strong reductant to reverse modification and restore enzyme activity.

Reactivation of Complex I. It is becoming increasingly apparent that certain oxidative modifications previously thought to be irreversible can be reversed by intracellular enzymatic systems indicating the potential for redox regulation (*56*−*65*). Evidence was sought for endogenous reactivation of complex I following Ca^{2+}/NADH-induced inhibition. As previously shown (Figure 4), following enzyme inhibition, the addition of EGTA to chelate Ca^{2+} was not sufficient for enzyme reactivation. Under the conditions of these experiments, NADH present during preincubation and complex I inhibition is consumed prior to addition of EGTA. In striking contrast to experiments in which EGTA was added alone (Figure 4), following inhibition of complex I, addition of EGTA and NADH resulted in complete reactivation of the enzyme (Figure 6). Thus, complex I can be reversibly inhibited by oxidative modification in response to alterations in Ca^{2+} concentration.

To further explore the chemical nature of complex I inhibition and reactivation, the ability of the thioredoxin/thioredoxin reductase/NADPH and glutaredoxin/GSH systems to restore enzyme activity was determined. These systems catalyze the reduction of protein disulfides and mixed disulfides with GSH, respectively. Following preincubation with Ca^{2+}/NADH and complex I inhibition, solubilized mitochondria were incubated with each disulfide reducing systems for 30 s prior to analysis of enzyme activity. As shown in Figure 7, both systems were capable of restoring enzyme activity. Addition of NADPH (10 μM) alone exhibited no effect. The formation of a protein mixed disulfide with GSH would be surprising given that experiments were performed utilizing solubilized mitochondria at 10.0 μg protein/mL. Therefore, GSH levels would be low relative to intact mitochondria, and rapid glutathionylation would not be expected. Additionally, treatment of GSH-depleted sub-mitochondrial particles with Ca^{2+}/NADH resulted in a similar degree of complex I inhibition (not shown). Importantly, addition of GSH (0.5 mM) after Ca^{2+}/NADH-induced inhibition of complex I largely prevented endogenous regeneration of complex I activity upon incubation with EGTA/NADH (Figures 6 and 7). GSH rapidly reacts with protein sulfenic acid residues and can react with protein disulfides to form mixed disulfides. Therefore, glutaredoxin/GSH-dependent reactivation of complex I (Figure 7) likely reflects reaction of exogenously added GSH with a sulfenic acid or disulfide on complex I followed by removal of GSH and regeneration of protein sulfhydryl by glutaredoxin. While reactivation of complex I with the thioredoxin system would indicate the involvememt of disulfide bond formation, this system has also been shown to reduce sulfenic acid. Thus, the exact nature of the Ca^{2+}/NADH-induced sulfhydryl modification to complex I cannot be conclusively established. These results indicate the potential for enzyme inhibition followed by glutathionylation in intact mitochondria where GSH is at concentrations sufficient for reaction with the modified sulfhydryl residue(s) on complex I. Depending on the duration of Ca^{2+}-induced enzyme inhibition, reactivation may proceed through a complex I-dependent mechanism or through the action of glutaredoxin.

DISCUSSION

Complex I of the mitochondrial respiratory chain has previously been shown to decline in activity during cardiac ischemia/reperfusion (*1*−*5*). The present in vitro study provides a potential mechanism of inhibition consistent with the cellular and mitochondrial environment that exists during ischemia/reperfusion. In addition, the reversible nature of these processes suggests the potential for regulation. Complex I was found to be susceptible to Ca^{2+}-mediated inhibition. Inhibition required the presence of NADH and was completely prevented by inclusion of superoxide dismutase but not catalase. Interaction of superoxide anion and pro-oxidants with sulfhydryl functional groups of protein cysteine residues can result in the formation of sulfenic acid derivatives and disulfide bonds. The thiol reducing agent DTT is capable of reducing each of these oxidative modifications, thereby regenerating cysteine (*52*−*55*). DTT was able to reverse inhibition of complex I, thereby indicating that Ca^{2+}-mediated NADH-dependent inhibition was due to oxidative modification of protein-associated sulfhydryl group(s). Importantly, upon removal of Ca^{2+}, oxidative inhibition can be reversed in the presence of the enzyme substrate, NADH. Little is known regarding the regulatory properties of complex I. These studies identify complex I as an enzyme that is oxidatively inhibited in a reversible fashion in response to alterations in Ca^{2+} concentration.

Previously, it has been demonstrated that complex I can be inactivated upon exposure to superoxide anion generated exogenously or to sulfhydryl reactive compounds such as oxidized glutathione (GSSG) or *N*-ethylmaleimide (*32*−*35*, *38*, *40*, *41*, *43*, *66*). Due to the reactive nature of these species, similar effects have been reported for numerous enzymes. It was therefore important to determine whether complex I undergoes inhibition and/or inactivation in response to oxygen radicals produced by endogenous processes. We provide evidence that inhibition of complex I occurs through the endogenous production of superoxide anion. Inhibition required turnover of complex I and occurred only in the presence of Ca^{2+}. Ca^{2+} has previously been shown to promote complex I inactivation by sulfhydryl reactive compounds (*34*, *35*, *43*). Therefore, in the current study, it is likely that Ca^{2+} induces structural alterations that expose a reactive cysteine residue(s) on complex I to superoxide anion produced at low levels during normal turnover of the enzyme. Due to the reversible nature of Ca^{2+}- and superoxide anion-dependent alterations in complex I activity, the contribution of this form of modification may be overlooked under certain conditions of ischemia/reperfusion, particularly upon analysis of mitochondria that are isolated in the presence of EDTA and/or EGTA and maintain a reductive potential. Ischemia/reperfusion induced deficits in activity which persist upon mitochondrial isolation likely reflect the

occurrence of downstream events such as depletion of the phospholipid cardiolipin (*38*, *67*).

Given the rapid and reversible nature of the oxidative inhibition of complex I, it is important to consider the possibility that this process represents an appropriate metabolic response necessitated by physiological and pathophysiological alterations in cytosolic and mitochondrial Ca^{2+} concentrations. Cardiac mitochondria serve to buffer cellular Ca^{2+} under conditions of Ca^{2+} overload such as cardiac ischemia/reperfusion (*11*−*13*) and exhibit increases in Ca^{2+} concentrations in response to normal Ca^{2+} transients (*68*−*72*). Ca^{2+} uptake by mitochondria is dependent on the proton gradient created across the inner mitochondrial membrane as a result of proton translocation by respiratory chain components (*13*−*16*). Ca^{2+}-induced inhibition of complex I may therefore alter the mitochondrial proton gradient to limit Ca^{2+} uptake, thereby preventing mitochondrial Ca^{2+} concentrations from reaching levels capable of inducing irreversible disruption of mitochondrial structure and function. Importantly, alterations in pH during ischemia and reperfusion may regulate this process by providing an indication of the relative necessity for mitochondrial Ca^{2+} buffering. If acidosis persists during reperfusion, a condition indicative of continued Ca^{2+} overload, complex I would remain active (Figure 2) enabling the accumulation of Ca^{2+} by the mitochondria. In contrast, if normal physiological pH is restored, the relative importance of mitochondrial Ca^{2+} uptake would be diminished. In this scenario, complex I would be inhibited, and thus, membrane potential and Ca^{2+} uptake would be reduced allowing for net Ca^{2+} efflux and eventual regain in complex I activity.

The maximum degree of complex I inhibition observed in this study was approximately 35%. As previously discussed (see Results), this may be an underestimation, given the nature of the enzyme assay and/or may reflect subtle regulation of this complex enzyme. Partial inhibition of the enzyme may be indicative of the delicate balance that must be maintained to simultaneously limit Ca^{2+} uptake while retaining a proton gradient sufficient to allow for ATP production and prevent opening of the permeability transition pore. Subtle alterations in membrane potential would be expected to alter the relative rates of Ca^{2+} uptake and efflux shifting the equilibrium between intramitochondrial and cytosolic Ca^{2+} concentration. The results of the current study provide the basis for future investigations required to determine the contribution of Ca^{2+}- and superoxide anion-dependent modulation of complex I activity to maintenance of Ca^{2+} homeostasis during normal physiological function and under pathophysiological conditions associated with Ca^{2+} overload.

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