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S-Nitrosation of Mitochondrial Complex I Depends on Its Structural Conformation*

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Nitric oxide is known to cause persistent inhibition of mitochondrial respiration as a result of *S*-nitrosation of NADH: ubiquinone oxidoreductase (complex I) (Clementi, E., Brown, G. C., Feelisch, M., and Moncada, S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 7631–7636). Little is known about whether such nitrosation occurs in physiological conditions and, if so, what are the possible cellular mechanisms. We have now found that the conformational state (active/deactive transition (Vinogradov, A. D. (1998) *Biochim. Biophys. Acta* 1364, 169–185)) of mitochondrial complex I is an important factor for the interaction of the enzyme with nitrosothiols and peroxynitrite. Only the deactivated, idle form of complex I was susceptible to inhibition by nitrosothiols and peroxynitrite. In contrast, the active form of the enzyme was insensitive to such treatment. Neither form of complex I was inhibited by nitric oxide itself. Our data suggest that the process of active/deactive transition plays an important role in the regulation of complex I activity and cellular respiration by nitric oxide. The implications of this finding for hypoxic or pathophysiological conditions *in vivo* are discussed.

Proton-translocating NADH:ubiquinone oxidoreductase (EC 1.6.5.3, complex I or type I NADH dehydrogenase) is the most complex enzyme of the mitochondrial respiratory chain. It is responsible for oxidation of matrix NADH and is the major entry point for electrons to the respiratory chain. Mammalian complex I consists of at least 45 subunits (1), of which more than 30 are so-called "accessory" subunits, not directly involved in catalysis, whose functions are still unclear. Some of them may have a structural role, assisting in the assembly of the enzyme (2), whereas others may be necessary for the fine regulation of the activity of the enzyme in response to signals from the cytoplasm and matrix.

Some years ago inhibition of complex I-mediated respiration was demonstrated in cells after incubation with activated macrophages (3). Following the discovery that nitric oxide (NO)[2] is generated endogenously and acts as a biological mediator (4), this inhibition of complex I activity was found to be due to NO (5). An inhibitory effect of the combined action of NO and superoxide anion on complexes I and II was demonstrated in preparations of intact mitochondria. This was shown to be due to peroxynitrite ($ONOO^-$), a compound that explains the NO-dependent inactivation of several components of the respiratory chain (6–8). Our group found that prolonged exposure to high concentrations of NO led to persistent inhibition of complex I, which we attributed to nitrosation of critical thiol residue(s) (9–11), a finding that has been confirmed by other groups (12–16). Although the degree of inhibition of complex 1 caused by various NO donors and the stoichiometry of the subsequent modification(s) have been investigated, the precise nature of the targeted subunit(s) and the possibility of nitrosation of complex I during physiological processes have not been established.

The catalytic properties of eukaryotic complex I are not simple (for a review see Ref. 17). Two catalytically and structurally distinct forms exist in any given preparation of the enzyme as follows: one is the fully competent, so-called "active" A-form and the other is the disabled, silent, "de-activated" D-form. So-called pseudoreversible A/D transitions have been described in mammalian complex I (18) and other eukaryotic complex I (19) and have been reviewed in detail (17, 20). After exposure of idle enzyme preparations (mitochondria (21), submitochondrial particles (SMP) (18), or purified complex I (22)) to elevated but physiological temperatures (>30 °C) in the absence of substrate, when catalytic turnover cannot occur, the enzyme converts to the D-form. In such a preparation there is a considerable lag phase during the continuous assay of the NADH: ubiquinone oxidoreductase reaction. Addition of a small (5–10 μM) pulse of NADH before the assay results in activation of the enzyme after several slow turnovers when NADH is oxidized by the quinone. This eliminates the lag phase, and the enzyme becomes fully active and catalyzes oxidation of NADH at a linear rate (18, 22). Therefore, the final zero-order steady state reaction rates for both forms of the enzyme are the same and depend only on the amount of the enzyme added. In the presence of divalent cations (Mg^{2+} and Ca^{2+}), or at alkaline pH, the activation takes longer, and the lag phase is more pronounced. Divalent cations have no effect on the activity of the A-form.

The high activation energy (270 kJ/mol) of the deactivation process (22) indicates the occurrence of major conformational

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[2] The abbreviations used are: NO, nitric oxide; $ONOO^-$, peroxynitrite; CPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DETA-NO, (*Z*)-1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]-diazen-1-ium-1,2-diolate; DTNB, 5,5′-dithiobis-(2-nitrobenzoate); DTT, dithiothreitol; GSNO, *S*-nitrosoglutathione; HAR, hexaammineruthenium(III) chloride; NEM, *N*-ethylmaleimide; Q_1, 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone; SIN-1, linsidomine; SMP, submitochondrial particles; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SOD, superoxide dismutase.

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changes in the organization of the enzyme. However, until now, the only conformational difference observed between these two forms is the number of cysteine residues exposed at the surface of the enzyme (23, 24). Treatment of the D-form of complex I with the sulfhydryl reagents NEM or DTNB irreversibly blocks critical cysteine residue(s), abolishing the ability of the enzyme to respond to activation, thus inactivating it irreversibly. The A-form of complex I is insensitive to sulfhydryl reagents. It has been shown that modification of a cysteine residue(s) of a single subunit with an apparent mass of 15 kDa is responsible for the irreversible inactivation of the D-form by NEM (23). In the A-form of complex I this subunit is not accessible to any covalent modification, accounting for the insensitivity of this form to NEM.

Despite advanced biochemical analysis of the kinetic behavior of both forms of complex I (20), the functional role of the observed heterogeneity has not yet been explained. We have now investigated the effect of nitrosothiols and $ONOO^-$ on the A- and D-forms of the enzyme. We have found that only the conformationally modified D-form of complex I is a target for nitrosothiols and $ONOO^-$, whereas the active form is insensitive to these compounds. Furthermore, we demonstrate that there are NO-dependent biochemical mechanisms that can influence the equilibrium between the A- and D-forms. It is possible that A/D transitions of complex I take place in certain pathophysiological situations, making this enzyme an early mitochondrial target for nitrosative stress.

MATERIALS AND METHODS

Bovine heart SMP were prepared according to standard procedure (18) and stored in liquid nitrogen. As predicted from earlier studies (25), around 40% of complex I in our preparation was present in the D-form because of the temperature conditions during purification.

To prepare SMP in which complex I is present almost entirely in the D-form, an aliquot of frozen membranes was thawed, diluted to 5 mg/ml with standard medium (including 0.25 M sucrose, 50 mM Tris-HCl (pH 8.5), 0.2 mM EDTA, 0.1 mM DTPA, 10 μM neocuprine supplemented with 1 mM malonate for activation of succinate dehydrogenase (26) and 5 mM $MgCl_2$), and incubated at 35 °C for 1 h. This treatment resulted in almost complete deactivation of complex I. The resulting SMP were diluted twice with the same buffer at pH 8.8 and incubated aerobically for 20 min at room temperature with 1% ethanol and 400 μM NAD^+ while being constantly stirred. To obtain SMP in which complex I is present in the A-form, alcohol dehydrogenase (100 μg/ml from *Saccharomyces cerevisiae*; Sigma) was added to the above mixture. During a 20-min incubation with alcohol dehydrogenase, the substoichiometric amounts of NADH produced by the regeneration system were oxidized by complex I; this treatment resulted in full activation and maintained the enzyme in the A-form when kept at 25 °C. Such treatment was found to be more efficient at preserving complex I in its A-form at physiological temperatures than the previously described incubation of SMP with slowly oxidized NADPH (23).

Oxidation of NADH was determined spectrophotometrically (using a Varian Cary 4000) as a decrease in absorption at 340 nm with 200 μM NADH in the standard assay medium (pH 7.0) containing SMP (10–25 μg of protein/ml). As additional measurements of complex I activity, NADH:Q_1 or NADH:HAR oxidoreductase reductase were assayed in the presence of 1 mM cyanide with the addition of 100 μM Q_1 or 2 mM HAR, respectively. If the measurement of full NADH:oxidase activity of the D-form of complex I was required, the enzyme was "activated" by a 10 μM pulse of NADH added directly to the spectrophotometric cuvette, and then full NADH oxidase or NADH:Q_1 reductase was assayed.

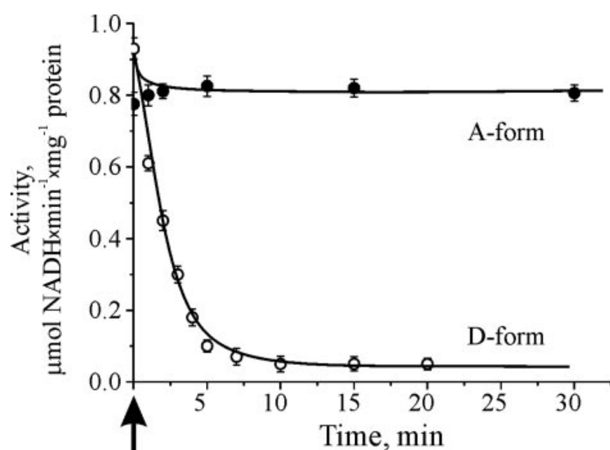

FIGURE 1. **Effect of NEM on NADH oxidase activity of deactivated (D-form) and activated (A-form) complex I in SMP.** SMP (2.5 mg/ml) were treated with 0.2 mM NEM at 15 °C in standard buffer (23). Addition of NEM at zero time is shown by an *arrow*. Aliquots were taken at the time specified, and NADH oxidase activity was assayed as described.

Solutions of GSNO, SNAP, DETA-NO, and SIN-1 were prepared freshly before the experiments. Peroxynitrite was synthesized according to a previously published protocol (27).

All chemicals were purchased from Sigma except SIN-1, which was from Invitrogen. Protein content was determined with a Biuret assay. Data are presented as means ± S.E. ($n > 3$). The experimental details are described in the legends to figures.

RESULTS

NADH Regeneration System; Sensitivity of A- and D-forms to NEM—Because of the fast thermally dependent spontaneous transition of the enzyme to the D-form, the maintenance of the enzyme in its active state at temperatures of >20 °C requires special conditions. An alcohol dehydrogenase/ethanol regeneration system for NADH (28) was therefore developed and successfully implemented to keep the enzyme in the A-form. The below-K_m concentrations of NADH generated by the metabolism of ethanol by alcohol dehydrogenase were continuously oxidized by complex I of the SMP, maintaining the enzyme at a constant turnover even at temperatures above 25 °C. Fig. 1 demonstrates the dramatic difference in sensitivity to NEM of the A- and the D-form of the enzyme. Addition of NEM had only a slight inhibitory effect on the NADH oxidase activity of the A-form, whereas it rapidly inhibited the enzyme activity of the D-form. Thus, upon deactivation, complex I appeared to become sensitive to cysteine modification.

Effect of Low Molecular Weight Nitrosothiols on Complex I—Fig. 2 demonstrates a dramatic difference in sensitivity of the A- and the D-form of complex I toward *S*-nitrosoglutathione (GSNO)

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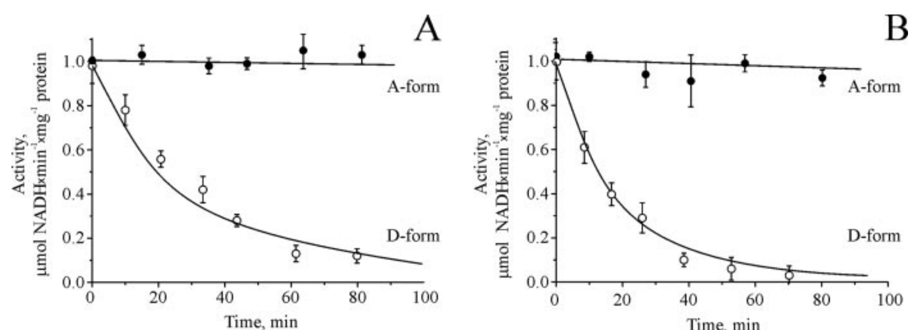

FIGURE 2. **Effect of the low molecular weight nitrosothiols GSNO (*A*) and SNAP (*B*) on NADH oxidase activity of the A-form and the D-form of complex I in SMP.** SMP (2.5 mg/ml) were treated as described under "Materials and Methods" and incubated with 1 mM nitrosothiol at 25 °C. Aliquots were taken during incubation, and NADH oxidase activity was assayed.

Table 1). We found that the addition of 1 mM CPTIO, a potent scavenger of NO ($k \sim 10^4$ M^{-1} s^{-1}) (29), did not change the inhibitory action of GSNO on the D-form of the enzyme. CPTIO alone has no effect on the activity of the D-form (not shown). In contrast with GSNO and SNAP, prolonged incubation (>2 h) of SMP with 2 mM DETA-NO did not affect the NADH oxidase activity of the D-form of complex I, neither did reduced nor oxidized glutathione (1 mM) (Table 1). Under the same experimental conditions, none of the above treatments had any effect on the A-form of the enzyme (not shown).

TABLE 1

Action of different effectors on the D-form of complex I

All conditions were as described in the legend to Fig. 2. The D-form of complex I in SMP (2.5 mg/ml) was incubated with different additions for 2 h at room temperature. No significant change in any of the NADH-dependent activities of control SMP was observed during incubation in these conditions.

	Complex I activity		
	NADH-oxidase	NADH:Q_1 reductase	NADH:HAR reductase
	µmol NADH × min^{-1} × mg^{-1}		
Control	1.10 ± 0.05	0.80 ± 0.07	6.8 ± 0.1
1 mM GSNO	0.15 ± 0.09	0.12 ± 0.09	6.7 ± 0.1
1 mM GSNO + 1 mM CPTIO	0.12 ± 0.05	0.11 ± 0.08	6.8 ± 0.0
2 mMDETA-NO	1.21 ± 0.02	0.83 ± 0.05	6.8 ± 0.1
1 mMGSH	1.11 ± 0.10	0.82 ± 0.10	6.8 ± 0.0
1 mMDETA-NO + 0.2 mM GSH	0.95 ± 0.09	0.79 ± 0.09	6.8 ± 0.0
1 mMGSSG	0.96 ± 0.10	0.75 ± 0.1	6.8 ± 0.0

TABLE 2

Reversal of the inhibition by GSNO of the D-form of complex I in SMP

SMP containing the D-form of complex I, incubated with GSNO as in Fig. 2, were washed twice (120,000 × *g* for 50 min) and resuspended in the same volume of measuring medium (pH 8.5) to obtain a concentration of 2.5 mg/ml (SMP-GSNO). The reducing agents were added and SMP were incubated at 25 °C for 2 h before the enzyme activity was determined.

	Complex I activity	
	NADH-oxidase	NADH:Q_1 reductase
	µmol NADH × min^{-1}× mg^{-1}	
GSNO alone	0.15 ± 0.05	0.12 ± 0.06
1 mM GSH	0.60 ± 0.09	0.55 ± 0.16
1 mM DTT	0.65 ± 0.14	0.73 ± 0.15
1 mM GSH + 0.2 mM $CuCl_2$	0.87 ± 0.09	0.70 ± 0.10
0.2 mM $CuCl_2$	0.18 ± 0.12	0.06 ± 0.10

or *S*-nitroso-*N*-acetylpenicillamine (SNAP) (Fig. 2, *A* and *B*, respectively). Only the D-form was inhibited by GSNO or SNAP, whereas the A-form was insensitive to these nitrosating agents. The inhibitory effect of GSNO on NADH oxidase activity was similar when NADH:Q_1 reductase activity was measured, indicating a direct effect on complex I and not on downstream components of the respiratory chain (Table 1). Furthermore, the oxidation of NADH by the artificial acceptor HAR was not affected by treatment with GSNO, showing that the content of complex I in the SMP was unchanged.

During decomposition low molecular weight nitrosothiols such as GSNO and SNAP can generate free NO in the solution. Complex 1 can be modified either by transfer of NO^+ or via direct nitrosation by the NO generated. To distinguish between these two mechanisms, additional assays were conducted (see Table 1).

Because *S*-nitrosothiols are sensitive to reducing agents, following treatment with GSNO the inhibited D-form of the enzyme was washed to remove excess GSNO, resuspended in the same medium, and treated with reducing agents. Addition of DTT (1 mM) or GSH (1 mM) led to a significant recovery of the NADH-dependent activities (Table 2). Copper ions are known to catalyze the fast decomposition of *S*-nitrosothiols, and the reversal of the inhibition was found to be greater when SMP were incubated together with copper and GSH (Table 2).

Effect of Peroxynitrite on Complex I—Peroxynitrite is known to nitrate or nitrosate amino acids such as tyrosine (30), to oxidize protein or non-protein thiols (31), and to cause inhibition of complex I in cell (9) mitochondria (7, 12) and SMP (13). The effect of $ONOO^-$ on the A- and D-forms of complex I is shown in Fig. 3*A*. Addition of $ONOO^-$ directly to the spectrophotometric cuvette had only a small effect on the NADH oxidase activity of the A-form, whereas that of the D-form was significantly inhibited (Fig. 3*A*). The succinate oxidase reaction was also affected by $ONOO^-$ but to a lesser extent (not shown). Addition of GSH (0.5 mM) to the buffer protected the D-form from inhibition by $ONOO^-$. Fully decomposed $ONOO^-$ had no effect on the activity of the enzyme (not shown).

Upon decomposition SIN-1 generates both superoxide anion and NO and is commonly used as a donor of $ONOO^-$. Addition of SIN-1 (0.5 mM) had no effect on the A-form but significantly inhibited the activity of the D-form of the enzyme (Fig. 3*B*). Addition of 200 units/ml bovine liver SOD in the incubation mixture protected the D-form from SIN-1-induced inhibition (Fig. 3*B*).

After washing, SMP treated with $ONOO^-$ or SIN-1 were subjected to incubation with the reducing agents shown in Table 2. Complex I inhibition was not reversed by prolonged (2–3 h) treatment with any of the reducing agents described (not shown), thereby indicating that the effect of $ONOO^-$ on the D-form of the enzyme was not simply dependent on *S*-nitrosation.

The key cysteine residue determining the sensitivity of the D-form of the enzyme to NEM can be modified by other SH reagents. As reported previously (24), only the D-form of complex I is inhibited by 5,5′-dithiobis-(2-nitrobenzoate), (DTNB, Ellman's reagent), but unlike NEM, the activity of the DTNB-

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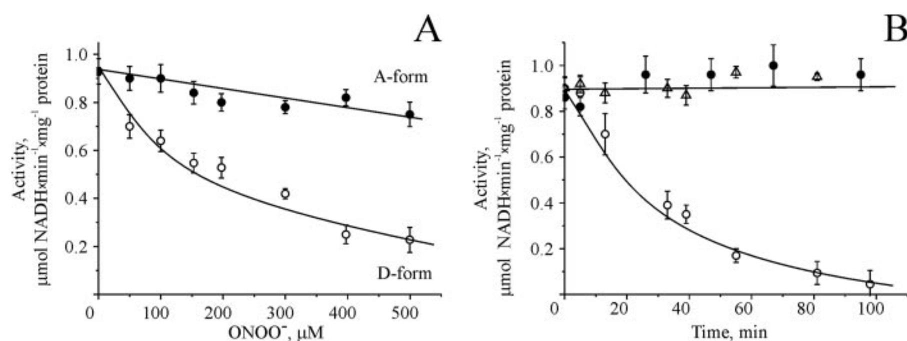

FIGURE 3. **Effect of peroxynitrite (ONOO$^-$, *A*) and the peroxynitrite donor SIN-1 (*B*) on NADH oxidase activity of the A-form and D-form of complex I.** SMP (2.5 mg/ml) were treated as described under "Materials and Methods" and either treated with a bolus of peroxynitrite added directly to the spectrophotometric cuvette prior to activity measurements (*A*) or incubated with 0.5 mM SIN-1 (*B*) at 25 °C. Aliquots were taken during incubation and NADH oxidase activity was assayed. *Triangles* indicate the effect of 200 units/ml bovine liver SOD on the D-form of complex I treated with SIN-1.

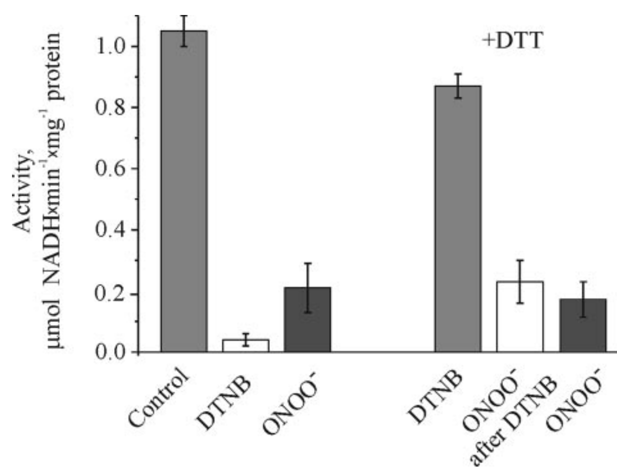

FIGURE 4. **Absence of a protective effect of DTNB against the irreversible inhibition of complex I by peroxynitrite.** SMP (1 mg/ml) in the D-form were incubated in standard buffer in the presence or absence of 0.5 mM DTNB for 5 min at 25 °C, pelleted by centrifugation, and resuspended in the same buffer. ONOO$^-$ treatment was then carried out as in Fig. 3. After ONOO$^-$ treatment, 2 mM DTT was added to remove DTNB, and after 2 min NADH oxidase activity was assayed as described. The inhibitory effect of DTNB alone was reversed by treatment with DTT, whereas that of peroxynitrite alone or following DTNB was not affected by the DTT.

inhibited enzyme can be restored after treatment with DTT. We investigated whether ONOO$^-$ targets the same cysteine residue by treating the D-form with DTNB and then with ONOO$^-$. Subsequent removal of the DTNB by adding DTT did not restore the enzyme activity, indicating that the effect of ONOO$^-$ cannot be explained simply by modification of the only critical cysteine involved in A/D transition (Fig. 4).

DISCUSSION

Our present results confirm previous observations on the inhibition of complex I by nitrosating agents (9, 12, 13, 15, 32). Furthermore, they show that the susceptibility of complex I to inhibition is dependent on the exposure of a crucial cysteine residue, which is characteristic of this protein in its de-activated form. The mechanism by which inhibition takes place may be through *S*-nitrosation, *S*-glutathionylation, or both (33). Protein *S*-nitrosation can result from a transnitrosation reaction, in which a nitrosonium ion is directly transferred from GSNO to another protein cysteine residue. Alternatively, the incubation of GSNO and a cysteine-containing protein can produce a mixed disulfide by a *S*-glutathionylation mechanism. However it has been shown that the glutathionylating activity of GSNO solutions can be explained by the formation of various decomposition products such as glutathione thiosulfinate or thiosulfonate (33, 34); therefore, it is not likely that freshly prepared GSNO solution is a good glutathionylating agent. Furthermore, *S*-glutathionylation (or *S*-thiolation in general) is a much slower process than *S*-nitrosation. Incubation of GSNO-inhibited complex I with glutathione transferase did not result in recovery of enzymatic activity (not shown). In addition, inhibition of complex 1 by SNAP, which is known to be a pure nitrosating agent (35), progresses at the same rate as that of GSNO. These observations, together with the fact that the inhibition by both agents was reversed by copper ions (Table 2) and that glutathione alone, whether reduced or oxidized, had no effect on either form of complex I, strongly support *S*-nitrosation as the mechanism of GSNO- and SNAP-induced inhibition.

In contrast, neither DETA-NO on its own nor SIN-1 in the presence of SOD was able to inhibit complex I. This, together with the fact that in the dark when chelating agents are present GSNO is fairly stable (*i.e.* does not release NO) and that its inhibitory action was not affected by CPTIO, suggests that the inhibition of complex I by GSNO or SNAP most likely occurs via a transnitrosation mechanism.

Peroxynitrite is a potent oxidant for various amino acid residues, with its highest reactivity toward cysteine (36). Our results show that ONOO$^-$ is a rapid and irreversible inhibitor of complex I activity, as has been reported for rat brain SMP (6). We were unable to see any reversal of the inhibition of ONOO$^-$ or SIN-1 by the reducing agents used. Furthermore, blocking of the crucial cysteine residue (as well as many other cysteines) of deactivated complex I with DTNB did not protect the enzyme from inhibition by ONOO$^-$. This suggests that inhibition of complex I by ONOO$^-$ may be caused by modification of other amino acid residue(s) or protein disulfide bond(s) besides the cysteine involved in the A/D transition.

Oxidation of NADH by the artificial acceptor hexaammineruthenium (HAR) was not affected by treatment with nitrosothiol agents. This is in agreement with previous results with NADH:ferricyanide reductase (8). Because the D-form of the enzyme is capable of catalyzing the NADH:HAR reductase reaction, we can assume that nitrosation of the D-form did not cause significant changes in the structure of the D-form peripheral domain of the enzyme or result in disruption of iron-sulfur clusters, and therefore complex I retains its integrity upon treatment.

In bovine heart SMP or mitochondria it is known that ~16 and 25 free SH groups per mg of protein, respectively, are avail-

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able for covalent modification, although not all of them belong to complex I (24, 37). Only 35% inhibition of complex I activity upon nitrosation of 10–15 cysteine residues of the enzyme has been found in mitochondria (16). This probably indicates that most of the modifications are not relevant for the catalytic properties of the enzyme. Although treatment of the A-form of the enzyme with nitrosating agents did not affect its catalytic properties, as functionally assessed by various activity measurements, this treatment undoubtedly resulted in modification of many cysteine-containing subunits. As shown before (23) and in this work, the A-form of complex I is insensitive to treatment even by millimolar concentrations of NEM. Most likely, the modification of almost all the surface-exposed SH groups in the active form of complex I by alkylation or nitrosation does not affect the physiological activity of the enzyme. This probably accounts for the minor degree of inhibition of complex I by GSNO (16) or $ONOO^-$ (38) in isolated mitochondria, where complex I is mostly present in its active form. However, the modification of only one cysteine residue of the transmembrane subunit ND3, which becomes exposed to the matrix side of complex I after deactivation, fully inhibits the enzyme.[3] This different sensitivity of the two forms of complex I to nitrosating agents could explain the difference in the degree of inhibition obtained by various research groups.

Although the existence of the A/D transition has been established in different preparations of complex I, the physiological relevance of this process has not been investigated. It is curious that at physiological temperatures the enzyme is required to expend some of the energy released in the NADH:ubiquinone reductase reaction to maintain a catalytically competent conformational state. It is possible that transition from the A- to D-form of complex I may take place during pathological conditions when the turnover of the enzyme is limited at physiological temperatures, such as during hypoxia or when the NO:oxygen ratio increases (metabolic hypoxia) (39). In such a situation the spontaneous deactivation of complex I would result in increasing sensitivity to nitrosating and oxidizing agents such as GSNO or $ONOO^-$, followed by blocking of the enzyme in the D-state. The details of these reactions have yet to be established.

Inhibition of complex I by *S*-nitrosation has been implicated in a number of pathologies, including Huntington, Alzheimer, and Parkinson diseases. Furthermore, decreased complex I activity has been reported in skeletal muscle biopsies taken from critically ill patients in septic shock (40). Ischemia-specific post-translational covalent modifications of several subunits of complex I have also been found recently after ischemia/reperfusion heart injuries (41), and the physiological occurrence of two forms of complex I and their interconversion during ischemia/reperfusion has been shown in studies *ex vivo* (42, 43). The inactivation of complex I may indeed contribute to the reduction of contraction of the heart during ischemic preconditioning, septic shock, and myocarditis (44). Because NO is capable of interacting with mitochondrially produced superoxide anion before the latter can be converted by SOD to hydrogen peroxide (45, 46), it is tempting to speculate that superoxide production in pathological conditions and in the presence of NO (47) would result in formation of $ONOO^-$ in the mitochondrial matrix followed by irreversible inhibition of the D-form of complex I that may accumulate during hypoxic conditions.

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