NADH Oxidase Activity of Mitochondrial Apoptosis-inducing Factor*

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Apoptosis-inducing factor (AIF) is a mitochondrial flavoprotein, which translocates to the nucleus during apoptosis and causes chromatin condensation and large scale DNA fragmentation. Here we report the biochemical characterization of AIF’s redox activity. Natural AIF purified from mitochondria and recombinant AIF purified from bacteria (AIFΔ1–120) exhibit NADH oxidase activity, whereas superoxide anion (O2−) is formed. AIFΔ1–120 is a monomer of 57 kDa containing 1 mol of noncovalently bound FAD/mol of protein. ApoAIFΔ1–120, which lacks FAD, has no NADH oxidase activity. However, native AIFΔ1–120, apoAIFΔ1–120, and the reconstituted (FAD-containing) holAIFΔ1–120 protein exhibit a similar apoptosis-inducing potential when microinjected into the cytoplasm of intact cells. Inhibition of the redox function, by external addition of superoxide dismutase or covalent derivatization of FAD with diphenylidionium, failed to affect the apoptogenic function of AIFΔ1–120 assessed on purified nuclei in a cell-free system. Conversely, blockade of the apoptogenic function of AIFΔ1–120 with the thiol reagent para-chloromercuriophenylsulfonic acid did not affect its NADH oxidase activity. Altogether, these data indicate that AIF has a marked oxidoreductase activity which can be dissociated from its apoptosis-inducing function.

Mitochondria are considered as central players in apoptosis of mammalian cells (1–3). Early during the apoptotic process, the outer mitochondrial membrane becomes permeabilized, and mitochondria release soluble proteins normally confined to the intermembrane space (4). Such apoptogenic proteins include the caspase activator cytochrome c (5), procaspases 2, 3, and 9 (6–8), the inhibitor of apoptosis protein (IAP) inhibitor Smac/DIABLO (9, 10), as well as AIF (11). In contrast to cytochrome c and Smac/DIABLO, AIF is a caspase-independent death effector, which translocates via the cytosol to the nucleus, where it causes chromatin condensation and large scale (50 kilobase pairs) DNA fragmentation (12, 13). Neutralization of the AIF protein by microinjection of a specific antibody into the cytoplasm of intact cells has revealed AIF to be rate-limiting for apoptotic chromatin condensation and, in some cases, for mitochondrial membrane permeabilization (11, 14, 15). Conversely, microinjection of AIF may cause full-blown apoptosis with nuclear condensation, dissipation of the mitochondrial transmembrane potential, release of cytochrome c, and exposure of phosphatidylserine on the outer plasma membrane leaflet (11, 14, 15).

The AIF precursor protein (612 amino acids) contains an N-terminal (first 100 amino acids) mitochondrial localization sequence. The protein is synthesized in cytoplasmic ribosomes and imported into the mitochondrial intermembrane space, where the mitochondrial localization sequence is cleaved off (11). The C-terminal domain of AIF (last 485 amino acids) shares significant homology with oxidoreductases from other vertebrates (Xenopus laevis, non-vertebrate animals (Caenorhabditis elegans, Drosophila melanogaster), plants, fungi, euubacteria, and archaeabacteria (16). The mature AIF protein purifies as a flavoprotein, both from mitochondria and from Escherichia coli used to produce recombinant AIF (11). This fact prompted us to investigate the putative electron transfer (redox) function of AIF, in relation to its apoptogenic activity. Indeed, apoptosis is accompanied by a general shift of the redox balance characterized by a depletion of NADH, NADPH, glutathione, as well as by an increase of free radicals, including superoxide anion, lipid peroxidation products (such as 4-hydroxynonenal), and oxidative damage of membranes and DNA (17). In several paradigms of apoptosis, culture in anoxic conditions, treatments with cell-permeable antioxidants, or overexpression of anti-oxidant enzymes (such as superoxide dis...

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1 The abbreviations used are: AIF, apoptosis-inducing factor; ΔΨm, mitochondrial transmembrane potential; DTNB, 5,5′-dithiobis-(2-nitrobenzene)-tetrazolium chloride; SOD, superoxide dismutase; DTT, dithiothreitol; HPLC, high performance liquid chromatography; Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; PAGE, polyacrylamide gel electrophoresis; DCPIP, 2,6-dichlorophenoldihydroxymethane.
mutase, glutathione peroxidase, catalase, the thioredoxin system) have profound inhibitory effects on cell death (18–20). It appears that the respiratory chain is a prime source for the generation of oxygen radicals (presumably derived from uncoupling and/or interruption of electron transfer due to the release of cytochrome c) (21). Moreover, mitochondrial targeting of anti-oxidant enzymes is particularly efficient in blocking apoptosis in several models (19, 22).

Here we report the detailed biochemical characterization of the redox function of AIF, which turns out to be a FAD-containing oxidase capable of oxidizing NAD(P)H while generating superoxide anion. Interestingly, the electron transfer function of AIF can be dissociated from its apoptogenic activity, both in cell-free systems and in intact cells.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 2 mM t-glutamine, 1 mM pyruvate, 100 mM Heps, 100 units/ml penicillin/streptomycin, and 10% decomplemented fetal calf serum (Life Technologies, Inc.). These cells were used for the purification of nuclear- and cell-free system experiments, as described (23). Rat-1 fibroblast cells were cultured as above and were used in microinjection experiments.

Recombinant AIF Proteins—AIF deletion mutant (Δ1–120) and AIF deletion mutant (Δ1–351) (11) were expressed from a Novagen pET32 expression vector and purified from E. coli. The proteins were stored at −80 °C in 50 mM Heps, pH 7.9, 100 mM NaCl, 2 mM EDTA, 1 mM DTT, and 10% glycerol.

Mass Spectroscopy, HPLC, and Elemental Analysis—Molecular mass was measured by means of a matrix-assisted laser desorption ionization system from Applied Biosystems at the Services Cientifico-Técnicos, Barcelona University. Sinapinic acid was used as matrix, and bovine serum albumin was used as standard protein. For the experimental analysis, AIFΔ1–120 (1.5 mg/ml) was mixed with sinapinic acid (10 mg/ml) in a 1:6 ratio of matrix to sample. Gel filtration was performed on a Superose 12 (Amersham Pharmacia Biotec) and was also used for molecular weight determination. The protein was eluted using 0.3 mM Heps, pH 8.2, 2 mM EDTA, 1 mM DTT, and 200 mM NaCl. Semiquantitative detection of metals was performed in an ELAN 6000 system (PerkinElmer Life Sciences), and semiquantitative determination of calcium was performed in a multicanal Thermo Jarred Ash 61E Polyscan following standard protocols.

SDS-PAGE—Proteins were run in a Phast System from Amersham Pharmacia Biotec, following the manufacturer’s instructions.

Free thiols’ content of AIFΔ1–120 preparations was determined by using a 50-fold molar excess of Ellman’s reagent, 5,5′-dithio-bis(2-nitrobenzoate)-(Nbs2) (DTNB) (24). The total thiols content was confirmed in the supernatant area and excess of NbsH2 (25). AIF was quantified spectrophotometrically on the basis of the extinction coefficient calculated in this work.

Absorption spectrometry studies were carried out using a Kontron Uvikon 860 spectrophotometer. The molar extinction coefficient of bound FAD at 450 nm was determined based on the absorption changes detected after releasing the bound FAD from the enzyme by heating (5 min at 90 °C) in 50 mM Tris-HCl, pH 8. An extinction coefficient of 11.3 M−1 cm−1 at 450 nm was assumed for the free FAD (26). Reductive titrations were performed under anaerobic conditions at 25 °C. The anaerobic enzyme sample, in 50 mM Tris-HCl, pH 8, was prepared in an anaerobic vial by sequential air evacuation and re-equilibration with oxygen-free argon. Anaerobic NAD(P)H was prepared identically, introduced by means of the titration syringe. Identical amounts of NAD(P)H were added to the reference cuvette.

HPLC for identification and quantification of FAD was performed using a C18 Vydac column. A linear gradient 0–100% of 0.1 mM ammonium acetate, pH 6, and methanol in 40 min was performed using 1 ml/min flow rate. FMN, riboflavin, and FAD were used as standards. 1 mg of AIFΔ1–120 in 1 ml of 50 mM Tris-HCl, pH 8, maintained in the dark, was heated for 10 min at 90 °C. After centrifugation, aliquots of the supernatant were injected into the HPLC, and flavins were detected at 445 nm.

Phosphorylated residues were determined by dot-blot analysis using mouse monoclonal anti-phosphoserine, anti-phosphothreonine, and anti-phosphotyrosine antibodies (Sigma) and an anti-mouse IgG alkaline phosphatase conjugate (Sigma). The method used was based on the procedure previously described (27), using polyvinylidene difluoride membranes (Immobilon-M from Millipore).

Visible redox titrations of AIF were performed under anaerobic conditions. Appropriate mediators that covered a potential range from +11 mV to −450 mV (1 mA) were added to the protein solution (10 μM) in 50 mM Bis-Tris for pH 6.5 or 100 mM Tris-HCl for pH values 7.5 and 9. Injection of small volumes of air-saturated sodium dithionite allowed the oxidation of the protein. A Crison 2002 digital potentiometer was used, and spectra were recorded on a Shimadzu UV-260 spectrophotometer. The reduction potential was determined by following the absorbance changes at 450 nm.

Potential involvement of cysteinyl redox centers in electron transfers was tested at 25 °C, under anaerobic conditions, following the NADH-Detection protocol described by Ohnishi et al. (28). Briefly, 18 μM AIFΔ1–120 in 50 mM sodium phosphate, pH 7, containing 0.5 mM EDTA was mixed with 0.5 mM NADH, 0.02% bovine serum albumin. The reaction was started adding 0.4 mM DTNB, and the nitroblue-azane anion production was monitored at 412 nm using an extinction coefficient of 13.6 mM−1 cm−1.

Aprotein preparation and hologen protein reconstitution AIFΔ1–120 apoprotein was prepared following the protocol described by Chapman and Reid (30) by exhaustive dialysis against 0.1 M Hepes, 2.5 mM CaCl2, 1 mM DTT, 0.1 mM EDTA, 0.1 M guanidine chloride, 17% glycerol (v/v) at pH 7.5, concentration on Centricon 30 K (Amicon) membranes, and a second round of dialysis against 50 mM Heps, 100 mM NaCl, 2 mM EDTA, 1 mM DTT, 10% glycerol at pH 7.9. The yield of this preparation was determined by ascorbate reductase activity. Hologen protein was performed by incubation with 1000-fold molar excess of FAD, acetone precipitation, and repeated ultracentrifugation on Centricon 30 K membranes to remove non-bound FAD.

Determination of AIF Redox Activities—Initial velocity studies of the NAD(P)H oxidase activity of the flavoprotein followed assay procedures described previously (31). Briefly, NAD(P)H oxidase activity was measured at 25 °C in a total volume of 0.5 ml containing 0.25 mM NADP in air-saturated 50 mM Tris-HCl, pH 8, buffer. The reaction was initiated by the addition of the enzyme and was followed by the decrease in absorbance at 340 nm. Steady-state kinetic data were obtained by varying NADH concentration. One unit of activity is defined as the amount of protein required to catalyze the conversion of 1 μmol of NAD(P)H to NAD(P)+ per minute at 25 °C. NBT reduction and monodehydroascorbate reductase activity (32, 33), superoxide formed in the reaction of AIFΔ1–120 with oxygen (34) and hydrogen peroxide production (35) were quantified as described. This latter reaction was carried out coupled with NADH oxidase in a mixture containing 1 mM sodium phosphate buffer, pH 6.9, 2.34 mg/ml phenol, 1 mg/ml 4-amanitotyrrine, and 0.02 units of horseradish peroxidase in a total volume of 0.5 ml. The absorbance was measured at 505 nm, and the concentration of H2O2 was calculated from a calibration curve (36). DCPIP (5 μM) or ferricyanide (2 mM) reduction were assayed as described (37). SOD inhibition was measured by adding the indicated units of enzyme to the reaction mixtures. The electron transfer between NADH-AIF-ferredoxin/adrenodoxin-cytochrome c was assayed as described (37), using AIFΔ1–120 instead of ferredoxin-NADP+ reductase and adrenodoxin instead of ferredoxin.

AIF Western Blot—Supernatants obtained from mitochondria undergoing permeability transition were subjected to a 10% SDS-PAGE and transferred (100 V, 5 min at room temperature) to a nitrocellulose membrane. AIF immunoblot analysis was performed using a rabbit antisem generated against a mixture of three peptides derived from the mouse AIF amino acids 151–200 (11).

In situ detection of 2, 2′-Di-nitrophenyl-5,5′-diphenyl-3,3′-(3′-3′-dimetioxy-4′-4′-difilen) tetrazolium chloride (NBT) reduction on native-PAGE was done using the reaction mixture described by Pez-Huertas et al. (32). Briefly, samples obtained from mitochondria undergoing permeability transition were loaded onto a 10% native-PAGE. The gel was incubated 20 min in the dark with 2 ml NBT solution. Then, 1 mM NADH was added to reduce NBT and the reaction was stopped with water after the appearance of the blue band.

Cell-free Systems of Nuclear Apoptosis—Purified HeLa cell nuclei (107/μl) were exposed 90 min at 37 °C to AIFΔ1–120 preincubated (15 min at 37 °C) with or without NADH, NADPH, para-chloromercuri-phenylhydulonic acid, superoxide dismutase, or diphenylenoiodium. For the last two assessments, assay were done using the same concentration, nuclei were preincubated with Hoechst 33342 (32 μM) for 15 min, 15 min, room temperature) and analyzed by fluorescence microscopy (Leica DM IRB). DNA content was determined by staining with propidium iodide (10 μg/ml) followed by analysis in a Vantage fluorescence-activated cell sorter (Becton-Dickinson). A minimum of 2500 events were scored.

Microinjection—Rat-1 fibroblasts were microinjected using a computer-controlled microinjector (pressure 150 hPa; 3 s; Eppendorf) with
FIG. 1. Absorption spectrum of recombinant AIF Δ1–120. The concentration used was 13 μM.

FIG. 2. NADH titration of AIF Δ1–120. The top line shows the spectrum of the oxidized enzyme (47 μM) before (a) and after addition of 0.12 (b), 0.35 (c), 0.59 (d), 0.83 (e), 1.07 (f), 1.31 (g), 1.57 (h), and 1.79 (i) equivalents of NADH/FAD. The inset shows the absorbance at 450 nm versus added NADH equivalents.

FIG. 3. Anaerobic titration of AIF Δ1–120 with an excess of NAD(P)H. A, long wavelength absorbance changes observed in oxidized AIF Δ1–120 (22 μM) without (a) or after addition of NADH (b and c; molar excess NADH/AIF was 3:1 (b) and molar excess was 6:1 (c), with maxima at 637 nm and 744 nm, respectively). B, same as A using NADPH instead of NADH, with appearance of maxima at 740 nm (b, molar excess NADPH/AIF; 3:1) and 769 nm (c, molar excess 6:1).

FIG. 4. pH effect on the redox potential of AIF Δ1–120. Titration curves obtained following absorbance variations at 450 nm of oxidized AIF Δ1–120 (10 μM) upon dithionite addition. Solid curves calculated from a dielectronic Nernst equation of −264 mV at pH 6.5 (A), −308 mV at pH 7.5 (B) and −373 mV at pH 9.0 (C).

FIG. 5. NAD(P)H oxidation by AIF Δ1–120. NADH (●) and NADPH (○) oxidation measured following absorbance variation at 340 nm, after addition of different amounts of NAD(P)H. AIF was added at a concentration of 3 μM (A). AIF Δ1–120 induced NBT reduction with NADH (●) and NADPH (○) as electron donors. AIF was added at a concentration of 85 nM (B).
buffer only, 7.5 μM AIFΔ1–120, apoAIFΔ1–120, FAD-reconstituted holoprotein, and AIFΔ1–351. After microinjection, cells were cultured at 37 °C for 180 min and stained with the DCm-sensitive dye CMXRos (100 nM, 15 min) and the DNA-intercalating dye Hoechst 33342 (1.5 μM, 15 min) (11). Microinjected viable cells (100/session, three independent sessions of injections) were identified by inclusion of 0.25% (w:v) FITC-dextran (green fluorescence) in the injectate. Only the blue and the red fluorescence were recorded.

All chemical reagents used in this work were purchased from Sigma.

RESULTS

Properties of Recombinant AIF Protein—Recombinant AIFΔ1–120 was found to elute as a single peak on a gel filtration column, with a calculated mass weight of 57 kDa, which corresponds to its theoretical molecular mass (57,046), indicating that, at near-physiological salt concentrations (200 mM NaCl), AIFΔ1–120 is a monomer. This result was confirmed by mass spectroscopy analysis (data not shown). It is in contrast with its apparent molecular mass determined by SDS-PAGE (67.5 kDa, about 10 kDa more than expected), a migration behavior reported for other proteins such as ferredoxins (38).

Table I

| Steady-state kinetic parameters of AIFΔ1–120 |
|---------------------------------------------|
| NADPH oxidase activity                      |
| Specific activity | \( k_{cat} \) | \( K_m \) | \( k_{cat}/K_m \) |
| units/mg | min\(^{-1}\) | μM | min\(^{-1}\) | μM |
| NADH   | 0.036 | 2.09 | 99.4 ± 10 | 0.021 |
| NADPH  | 0.098 | 2.84 | 52.9 ± 12 | 0.054 |

| NBT reductase activity                      |
|---------------------------------------------|
| Specific activity | \( k_{cat} \) | \( K_m \) | \( k_{cat}/K_m \) |
| units/mg | min\(^{-1}\) | mM | mM | min\(^{-1}\) | mM |
| NADH   | 38.733 | 2244.2 | 2.2 ± 0.3 | 1020  |
| NADPH  | 0.383  | 22.1   | 15.0 ± 0.1 | 14.8  |

| Superoxide production                       |
|---------------------------------------------|
| Specific activity | \( k_{cat} \) | \( K_m \) | \( k_{cat}/K_m \) |
| units/mg | min\(^{-1}\) | μM | μM | min\(^{-1}\) | μM |
| NADH   | 5.559 | 322.1 | 2.2 ± 0.2 | 146.4 |
| NADPH  | 0.79  | 45.6   | 15.0 ± 0.1 | 30.4  |

| Peroxide production                         |
|---------------------------------------------|
| Specific activity | \( k_{cat} \) | \( K_m \) | \( k_{cat}/K_m \) |
| units/mg | min\(^{-1}\) | μM | μM | min\(^{-1}\) | μM |
| NADH   | 0.462 | 26.7   | 19 ± 3 | 1.4  |
| NADPH  | ND    | ND     | ND     | ND   |

| Diaphorase activity (DCPIP)                 |
|---------------------------------------------|
| Specific activity | \( k_{cat} \) | \( K_m \) | \( k_{cat}/K_m \) |
| units/mg | min\(^{-1}\) | mM | mM | min\(^{-1}\) | mM |
| NADH   | 0.375 | 21.6   | 3.4 ± 0.4 | 6.3  |
| NADPH  | ND    | ND     | ND     | ND   |

\( ^{a} \) ND, not detected.

Fig. 6. NADH (●) and NADPH (○) oxidase activities of AIF result in generation of superoxide anion (A) and hydrogen peroxide (B). AIF concentration was 95 nM in A and 3 μM in B.

Fig. 7. A, cytochrome c reduction mediated by superoxide anion, using NADH as electron donor. B, specific inhibition of superoxide anion-mediated cytochrome c reduction by SOD. C, adrenodoxin enhances cytochrome c reduction. AIF concentrations were 72 nM in A and B, and 90 nM in C. NADH concentration was 3.4 mM in B and C.

Fig. 8. AIF is the dominant NADH oxidase released from mitochondria undergoing permeability transition. A, AIF release induced by 100 μM Ca\(^{2+}\) and 2 mM sodium arsenite measured by \( ^{15} \)NBT detection. B, AIF detection by immunoblot of mitochondrial intermembrane proteins. As a control of permeability transition induction, mitochondria were pretreated with 1 mM cyclosporine A (CycloA) to inhibit the release of the intermembrane mitochondrial proteins.

All chemical reagents used in this work were purchased from Sigma.

RESULTS

Properties of Recombinant AIF Protein—Recombinant AIFΔ1–120 was found to elute as a single peak on a gel filtration column, with a calculated mass weight of 57 kDa, which corresponds to its theoretical molecular mass (57,046), indicating that, at near-physiological salt concentrations (200 mM NaCl), AIFΔ1–120 is a monomer. This result was confirmed by mass spectroscopy analysis (data not shown). It is in contrast with its apparent molecular mass determined by SDS-PAGE (67.5 kDa, about 10 kDa more than expected), a migration behavior reported for other proteins such as ferredoxins (38).
AIF1–120 is non-covalently bound and was identified by HPLC as FAD (data not shown). FAD is the only identified prosthetic group present in AIF1–120, at a molar ratio of 1:1. The absorption spectrum of AIF (Fig. 1) shows the typical features of an oxidized FAD flavoprotein, with the visible maximum at 378 nm and 450 nm and a shoulder at 467 nm. The ratio $A_{378}$/$A_{450}$ was 7 in pure preparations, and the extinction coefficient for oxidized AIF at 450 nm was calculated to be 12.12 $\text{mM}^{-1} \text{cm}^{-1}$.

**Electron Transfer Reactions from NADH and NADPH to AIF**—The addition of an equimolar amount of NADH to AIF1–120 in anaerobic conditions leads to complete flavin reduction, without intermediate semiquinone formation (Fig. 2). Similar titration curves were obtained using NADPH as reductant, and in both cases the spectral changes are similar to the reduction of AIF1–120 with dithionite (data not shown). The reduced form was stable over several hours, and admission of air to the sample did not lead to the immediate appearance of the oxidized AIF spectrum. Upon addition of an increasing molar excess of NADH or NADPH over AIF1–120, the appearance of long wavelength absorbance bands was observed (Fig. 3). These long wavelength absorbances of the reduced enzyme were stable at 25 °C for hours, even upon exposure to air. They exhibit the blue-green and green color described for other electron transfer complexes (31). By analogy to other enzymes (31), these long wavelength absorptions are likely to correspond to charge transfer complexes between the reduced FAD and tightly bound NAD$^+$ or NADP$^+$.

**Redox Potential of AIF**—The spectral titration of AIF1–120 with dithionite revealed that the redox potential of AIF is strongly influenced by the pH (Fig. 4). Assuming a two-electron reduction step, midpoint redox potentials were determined to be $-264 \pm 15$ mV at pH 6.5 (Fig. 4A), $-308 \pm 15$ mV at pH 7.5 (Fig. 4B), and $-378 \pm 15$ mV at pH 9.0 (Fig. 4C). Neither semiquinone formation nor long wavelength absorbing bands were detected upon reduction by dithionite. A plot of AIF’s redox potential (FAD/FADH$_2$) versus pH has a slope of $-44$ mV (data not shown). This slope deviates from that expected for a two-electron reduction involving two protons (58 mV/pH unit) or one proton (29 mV/pH unit). The deviation from theoretical values indicates the possible presence of other dissociable groups whose $pK_a$ values are linked to the redox state of the enzyme. Even though titrations with dithionite and NAD(P)H showed an uptake of two electrons, it was considered important to discard the involvement of cysteinyl residues as active redox acceptors; in the NADH:DTNB oxidoreductase assay, no involvement of cysteinyl residues in reductase transfers was detected (data not shown).

**AIF Redox Activities**—AIF1–120 was found to exhibit NADH and NADPH oxidase activities (Fig. 5A). NADPH oxidation in presence of AIF was followed measuring initial rates of $\Delta A_{340}$ nm. The apparent $K_m$ for NADH was calculated as $99.4 \pm 10$ $\mu$M and the turnover number 2.09 min$^{-1}$. When NADPH was used as electron donor, the apparent $K_m$ was $52.9 \pm 12$ $\mu$M and the turnover number 2.8 min$^{-1}$ (Table I). These kinetic parameters are very similar to previous values described for other superoxide forming NADH oxidases (39), and the steady-state kinetic data may be interpreted taking into account the possible formation of relatively stable charge-transfer complexes. Addition of exogenous FAD did not stimulate the NADH oxidase activity of AIF1–120 (data not shown), in contrast to several NADH oxidases from bacteria (29, 35, 40–42). When oxidizing NADPH or NADPH, AIF catalyzed the reduction of the tetrazolium salt NBT (Fig. 5B). This was due to the AIF/FADH$_2$-mediated reduction of O$_2$ to O$_2^-$ (Fig. 6A). Accordingly, the reduction of NBT was completely abolished by

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**Fig. 9. Apoptotic activity of apoAIF1–120 and reconstituted holoAIF1–120 in a cell-free system and in microminection assays.** Spectral characteristics of apoAIF and reconstituted holoAIF are shown in A. The inset shows the recovery of the NADH oxidase activity of apoAIF after FAD reconstitution measured by in situ NBT detection (as in Fig. 8A). Rat-1 cells were microinjected with the indicated protein (7.5 $\mu$M AIF1–120, apoAIF1–120, and reconstituted holoAIF1–120), and stained with Hoechst 33342 (blue fluorescence) and the $\Delta \psi_m$ sensitive dye CMXRos (red fluorescence). Representative phenotypes obtained 3 h after injection are shown in B. Quantification of nuclear apoptosis and $\Delta \psi_m$ reduction induced by microinjection (100–150 Rat-1 cells/session) of AIF1–120, apoAIF1–120, reconstituted holoAIF1–120, or AIF1–351 (determined as in B) (C; $x \pm$ S.E. of three experiments).
the O$_2^-$ scavenger superoxide dismutase (SOD, 40 units/ml). Hydrogen peroxide formation resulting from superoxide production was also measured (Fig. 6B). AIF did not exhibit any peroxidase activity (data not shown).

AIFΔ1–120 was also found to possess a monodehydroascorbate reductase activity with a specific activity of 8.8 units/mg protein. DCPIP, a two-electron acceptor, with a $k_{cat}$ of 0.505 min$^{-1}$, a feature that is common to several NADH oxidases. The specific activity of AIF$\Delta1–120$ was able to reduce NADH, a two-electron donor, AIF in the presence or absence of 2 mM NADH or NADPH measured as in B, D, comparison of AIF redox and apoptotic activity obtained after pretreatment of AIFΔ1–120 (100 $\mu$g/ml, 15 min at 37°C) with NADH (2 mM), NADPH (2 mM), SOD (40 units/ml), diphenyleneiodonium (DPI, 250 $\mu$M), or para-chloromercuriphenylsulfonic acid (PCMPS, 30 $\mu$M) ($x \pm$ S.E. of five experiments).

In summary, AIF exhibits NADH oxidase activity and is able to transfer one electron (to molecular oxygen and ferricyanide) or two electrons (to DCPIP), as summarized in Table I. AIF Is the Dominant NADH Oxidase Released through the Mitochondrial Outer Membrane—Purified mitochondria can be induced to undergo the so-called permeability transition, a manipulation that leads to osmotic swelling of the matrix and physical rupture of the outer membrane, causing the release of soluble intermembrane proteins (4). Upon induction of permeability transition with Ca$^{2+}$ or arsenite, immunodetectable AIF was found in the mitochondrial supernatant. The release of AIF was inhibited by cyclosporin A, a specific inhibitor of the permeability transition pore (Fig. 8). Separation of proteins via native PAGE, followed by the in situ detection of an NADH oxidase activity causing the reduction of NBT, yielded one single blue band (Fig. 8A). This band also reacted with a specific anti-AIF antiserum and co-migrated with recombinant holoAIFΔ1–120 (Fig. 8B). Altogether, these data confirm that natural (mitochondrial) AIF possesses a NADH oxidase/NBT reductase activity causing the reduction of NBT, yielding one single blue band (Fig. 8A). This band also reacted with a specific anti-AIF antiserum and co-migrated with recombinant holoAIFΔ1–120 (Fig. 8B). Altogether, these data confirm that natural (mitochondrial) AIF possesses a NADH oxidase/NBT reductase activity causing the reduction of NBT, yielding one single blue band (Fig. 8A). This band also reacted with a specific anti-AIF antiserum and co-migrated with recombinant holoAIFΔ1–120 (Fig. 8B).
loAIF had a NADH oxidase-NBT reductase activity indistinguishable from native AIFΔ1–120 (Fig. 9A). Unmanipulated AIFΔ1–120, apoAIFΔ1–120, and reconstituted holoAIFΔ1–120 were micro-injected into the cytoplasm of Rat-1 fibroblasts. All three protein preparations induced a similar level of nuclear chromatin condensation, as well as a dissipation of the mitochondrial transmembrane potential (Fig. 9, B and C). A deletion mutant of AIFΔ31–351, which lacks part of the oxidoreductase domain, yielded negative results in this system (Fig. 9C). The nuclear effects of AIF were recapitulated in a cell-free system, in which AIFΔ1–120 was added to purified HeLa nuclei. In such a system, AIFΔ1–120 caused marked peripheral chromatin condensation (Fig. 10A) as well as a loss in DNA content (Fig. 10B). Addition of NADH or NADPH failed to enhance the apoptogenic activity of AIFΔ1–120 (Fig. 10C). Moreover, inhibition of the oxidoreductase activity by external addition of SOD or diphenyleneiodonium, an inhibitor of flavonoid-containing enzymes covalently reacting with FAD (43–45), failed to modify the apoptogenic activity of AIF (Fig. 10D). In contrast, addition of para-chloromercuriphenylsulfonic acid, a thiol-reactive agent, did abolish the apoptogenic activity of AIFΔ1–120, yet did not affect its NBT reductase activity (Fig. 10D).

Altogether, these data show that the apoptogenic and oxidase functions of AIF can be dissociated.

**DISCUSSION**

The results from this work indicate that AIF has a marked NADH oxidase activity. According to the classification by Masse-ey (46), AIF may belong to the electron-transferase class of NADH reductases, because it reacts rapidly with oxygen, forming O$_2^{-}$ and the flavoprotein neutral radical as products. No semiquinone intermediate could be detected, although such a neutral radical is expected to be produced after the transfer of a single electron. In each of the activities described, the reaction could be initiated by the reduction of AIF by the two-electron donors NADP/PH, in the absence of exogenous electron acceptor. The reduced form of AIF reacted with one-electron (molecular oxygen and ferricyanide) or two-electron (DCPIP) acceptors. Kinetic analysis of the AIF steady-state data are rather difficult to interpret, given the formation of relatively stable charge transfer complexes.

Several NADH oxidases from bacterial sources have been isolated and characterized (31, 35). The putative role of those enzymes is to maintain the cellular redox balance under aerobic conditions, by converting NADH to NAD$^+$. Additionally, several poorly characterized superoxide (O$_2^{-}$) forming NADH oxidases have been detected in animal cells, namely in heart mitochondria (48), liver mitochondria (49), microsomes from bovine myocytes (50), and endothelial cells (51). However, AIF is not similar to any of the previously described NADH oxidases. First, it is monomeric, whereas bacterial NADH oxidases are usually dimeric or tetrameric (52). Second, AIF transfers electrons without the involvement of cysteinyl groups, whereas other NADH oxidases rely on a redox-active disulfide center constituted by two vicinal cysteine residues (35, 42). Third, AIF oxidizes NADH via a mechanism that does not require the addition of exogenous FAD, whereas several NADH oxidases from bacteria do require FAD (29, 35, 40–42). Fourth, in contrast to several NADH oxidases (42), AIF does not function as a hydrogen peroxide scavenger.

AIF protein is present in the mitochondria of all mouse tissues that have been assessed and has also been found in a panel of 60 human cancer cell lines (53), suggesting that AIF may fulfill important metabolic functions. However, based on the present data, it is difficult to understand what the physiological function of AIF in normal (non-apoptotic) conditions may be. Since AIF is the only NADH oxidase detected in the intermembrane space, it is tempting to speculate that AIF accounts for the mitochondrial superoxide anion or hydrogen peroxide-generating NADH oxidase activity (54, 55), which is lost from mitochondria, once cells have been induced to die (55). Clearly, an NADH oxidase activity causing the collateral generation of superoxide anion radicals would be of no advantage for the cell. It thus may be speculated that the true, yet-to-be-discovered substrates of the AIF oxidoreductase compete for endogenous NADH and/or that AIF is normally inactivated by local inhibitory factors within the intermembrane space. If AIF acted as a superoxide-generating NADH reductase outside of mitochondria, after its apoptotic release, what might be the contribution of this enzymatic activity to the apoptotic process? Apoptosis is notoriously associated with a massive depletion of NADH/NADPH (56), as well as an increase in the generation of superoxide anions (57), at both mitochondrial and extramitochondrial localizations (17). Furthermore, it should be mentioned that, in isolated mitochondria, the permeability transition pore complex is tightly regulated by the oxidation-reduction state of the pyridine nucleotide pool, with oxidation causing an increase in the pore opening probability (58). Although these changes in the redox potential may be explained by a variety of factors, including uncoupling/blockade of the respiratory chain (21) and activation of poly(ADP)ribose polymerase (59), it will be interesting to study the contribution of AIF to this process, for instance in embryonic stem cells in which the AIF gene is ablated.

It appears that the known apoptogenic functions of AIF and its novel oxidoreductase activity can be dissociated from each other, based on three arguments. First, removal of the prostatic FAD group (which obviously abolishes the oxidoreductase function of AIF) does not curtail the apoptogenic effects of AIF on mitochondria and nuclei of microinjected cells. Second, addition of NADH, addition of SOD, or covalent derivatization of FAD failed to modulate the capacity of AIF to induce nuclear apoptosis in a cell-free system. Third, inhibition of the apoptogenic effect of AIF by means of para-chloromercuriphenylsulfonic acid failed to affect its NADH oxidase activity. These data are similar to those obtained for cytochrome c in the sense that the apoptogenic activity of cytochrome c does not depend on its redox status. Exchange of the Fe$^{2+}$ by Co$^{2+}$ within the heme prosthetic group of cytochrome c (a manipulation that abolishes the electron transfer function of heme) fails to alter its caspase-activatory functions (60), whereas certain amino acid substitutions that do not affect its redox function do abrogate cytochrome c-mediated caspase activation (60, 61). In conclusion, both cytochrome c and AIF thus appear to be bifunctional molecules with clearly dissociable redox and apoptogenic activities.

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**REFERENCES**

1. Green, D. R., and Kroemer, G. (1998) Trends Cell Biol. 8, 267–271
2. Green, D. R., and Reed, J. C. (1998) Science 281, 1399–1402
3. Kroemer, G., and Reed, J. C. (2000) Nat. Med. 6, 513–519
4. Patterson, S., Spahr, C. S., Daugas, E., Susin, S. A., Irinopoulos, T., Koehler, C., and Kroemer, G. (2000) Cell Death Differ. 7, 137–154
5. Budijardjo, I., Oliver, H., Lutter, M., Luo, X., and Wang, X. (1999) Annu. Rev. Cell Dev. Biol. 15, 269–290
6. Mancini, N., Nicholson, D. W., Roy, S., Thornberry, N. A., Peterson, E. P., Casciola-Rosen, L. A., and Rosen, A. (1998) J. Biol. Chem. 273, 1485–1495
7. Krajewska, S., Krajewska, M., Ellerby, L. M., Welsh, K., Xie, Z. H., Deveraux, Q. L., Salvesen, G. S., Bredesen, D. E., Essenthal, R. E., Fiskum, G., and Reed, J. C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5752–5757
8. Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Larochette, N., Ailhaud, P. M., and Kroemer, G. (1999) J. Exp. Med. 189, 381–394
9. Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000) Cell 102, 32–42
10. Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J., and Vaux, D. L. (2000) Cell 102, 43–53
11. Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jaccotet, E., Costantini, P., Loefller, M., Larochette, N., Goodlett, D. R., Aebsholds, R., Siderovski, D. P., Penninger, J. M., and Kroemer, G. (1999) Nature 397, 441–446
12. Daugas, E., Susin, S. A., Zamzami, N., Ferri, K., Irinopoulos, T., Larochette,
NADH Oxidase Activity of Mitochondrial Apoptosis-inducing Factor
M. Dolores Miramar, Paola Costantini, Luigi Ravagnan, Ligia M. Saraiva, Delphine Haouzi, Greg Brothers, Josef M. Penninger, M. Luisa Peleato, Guido Kroemer and Santos A. Susin

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