Redox-dependent Changes in Molecular Properties of Mitochondrial Apoptosis-inducing Factor*

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Mitochondrial apoptosis-inducing factor (AIF) is a central player in the caspase-independent cell death pathway whose normal physiological function remains unclear. Our study showed that naturally folded mouse AIF very slowly reacts with NAD(P)H (k_{cat} of 0.2-0.01 s^{-1}) forming tight, dimeric, and air-stable FADH$_2$-NAD(P) charge-transfer complexes ineffective in electron transfer. FAD reduction is accompanied by a conformational change involving AIF-specific N-terminal and regulatory 509–559 peptides and the active site His$^{453}$, and it affects susceptibility of AIF to calpain and AIF-DNA interaction, the two events critical for initiating caspase-independent apoptosis. Based on our results, we propose that formation of long-lived complexes with NAD(P)H and redox reorganization may be functionally important and enable AIF to act as a redox-signaling molecule linking NAD(P)H-dependent metabolic pathways to apoptosis.

Apoptosis-inducing factor (AIF)$^2$ is a highly conserved, phylogenetically old mitochondrial flavoprotein implicated in embryonic development, cardiac cell survival, carcinogenesis, and neurodegenerative disorders. In the late 1990s, AIF was identified as a key player in the caspase-independent cell death pathway (1, 2). These early studies suggested that mature AIF consists of residues 102–612 (Δ1–101), resides in the intermembrane space (IMS) of normal mitochondria and, upon an apoptotic stimulus, translocates to the nucleus and triggers chromatin condensation and large scale DNA fragmentation by cooperating with other proteins such as endonuclease G and cyclophilin A (CypA) (1, 3, 4). Recently, it has been proven that mature AIF starts at residue 54 and is anchored to the inner mitochondrial membrane (5), whereas the apoptogenic Δ1–101/102 form is produced upon proteolytic processing involving calpain I and cathepsins B, L, and S (6, 7).

Although the apoptotic function of AIF has been extensively studied (reviewed in Ref. 8), the role of the protein in normal mitochondria remains unclear. To date, functional and structural studies have been carried out mainly on the Δ1–120 and shorter forms of recombinant AIF, which were purified from bacteria as apoproteins and refolded to incorporate FAD (1, 9–11). Based on the redox properties and structural similarity to the disulfide reductase family of enzymes, AIF was proposed to function as a superoxide-generating NADH oxidase (9) or electron transferase (10). Other studies indicated that AIF might act in mitochondria as an antioxidant (12–14) or decrease reactive oxygen species levels indirectly by assisting biogenesis/maintenance of respiratory complexes I and III (15–17) and regulating glutathione levels (18). Finally, a recent observation that mitochondria in AIF-deficient neurons were fragmented, with aberrant cristae, led to a suggestion that AIF participates in maintaining mitochondrial structure (19). Despite the fact that there is a strong indication that AIF is linked to free radical homeostasis and oxidative stress, the mechanism and precise role of AIF in mitochondria remain elusive.

One problem in elucidating the physiological function of AIF is that only truncated and refolded forms of the recombinant protein have been investigated (9–11, 20, 21). In this study, molecular and redox properties of naturally folded mature and apoptotic AIF have been characterized and compared. Both flavoproteins were found to slowly react with NAD(P)H forming dimeric, air-stable FADH$_2$-NAD(P) charge-transfer complexes (CTCs) with notable differences in the kinetics of NAD(P)H dissociation and CTC decay imposed by dissimilarities in the length of the N terminus. The redox-linked monomer-dimer transition, similarly occurring in the mitochondrial protein, was accompanied by a conformational change that involved a unique 509–559 insertion, a potential protein-protein interaction site, and affected susceptibility of AIF to proteases and AIF-DNA interaction, critical for initiation of the caspase-independent apoptotic pathway. Our results challenge previous concepts and suggest that AIF is a redox-signaling molecule whose normal and apoptotic functions are controlled by NAD(P)H.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The cDNA of mouse AIF (I.M.A.G.E. Consortium Clone 2225457) was used as a template to clone the Δ1–53 and Δ1–101 forms of the protein into a pKK233–3 vector with a cleavable C-terminal 6-histidine affinity tag. Proteins were expressed in Escherichia coli strain BL21(DE3) and purified as described in the supplemental mate-
Interaction of AIF with NAD(P)H

Kinetics of AIF reduction with NAD(P)H was monitored under anaerobic conditions at 452 nm and 25 °C using a SX.18MV stopped-flow spectrophotometer (Applied Photophysics, UK). Anaerobic samples were prepared as described previously (22). Redox potentials of AIF were determined spectrophotometrically in xanthine saturated with subequimolar NAD(P)H, and purged with pure oxygen to initiate flavin oxidation that was monitored at 452 nm and 25 °C. CD spectra of 2.5–5 μM AIF were monitored at 452 nm and 25 °C using a J-720 spectropolarimeter.

Determination of the Molecular Size of AIF—The molecular size of recombinant AIF was determined by gel filtration on Superdex-200 (Amersham Biosciences) in 50 mM phosphate, pH 7.0, 0.5–10 nmol of AIF in the presence or absence of a 20-fold excess of NAD(P)H were recorded in 12 mM phosphate, pH 7.0, using a 0.1-cm path length cuvette and a Jasco J-720 spectropolarimeter.

Proteolytic Digests—10 μM AIF in 20 mM Tris-HCl, pH 7.5, 2 mM CaCl2 was incubated with trypsin (0.06 mg/ml, Sigma) or human calpain I (5 units/ml, Calbiochem) at 37 °C in the absence and presence of 2 mM NAD(P)(H) for 15 min at 25 °C, where position of the absorption maximum reflects affinity of the complex: the greater the interaction between the reacting molecules, the longer the wavelength of the CTC absorption maximum (320 mV).

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Interaction of AIF with NAD(P)H—Interaction of AIFΔ1–101 with linearized double-stranded DNA was examined using gel-retardation assays (25). 1–100 μg of AIF was incubated with a 100-bp DNA ladder (180–250 ng, New England Biolabs or Invitrogen) for 15 min at room temperature in 20 mM Hepes, pH 7.0, in the absence and presence of 2 mM NAD(P)(H) and equimolar amounts of recombinant human CypA (Calbiochem) or heat shock protein 70 (Hsp70) and electrophoresed in 50 mM Tris acetate on 1.2–2% agarose gel containing 0.6 μg/ml ethidium bromide. The ability of AIFΔ1–101 to degrade DNA was examined as described by Cande et al. (4). 20 nM AIF was incubated with 200 ng of a pNEB plasmid (2.7 kb, New England Biolabs) with or without 20 mM CypA for 1–4 h at 37 °C. The resulting mixtures were resolved on 1.2% agarose gels and visualized with ethidium bromide.

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AIF Release from Mitochondria—Mice liver mitochondria were isolated by standard differential centrifugation. Protein concentrations were measured by the Pierce BCA method. AIF release was induced as described previously (6, 7). Briefly, 400 μg of mitochondria was incubated for 15 min at room temperature in the respiration medium (125 mM KCl, 2 mM K2HPO4, 20 mM HEPEs, pH 7.0, 4 mM MgCl2, 3 mM ATP, 1 mM ADP, 1 μM ruthenium red) containing either 5 mM malate/glutamate or 5 mM succinate and 2 mM rotenone with or without 0.5–5 mM NAD(P)(H) and 100 μM calpeptin (Calbiochem), then placed for 60 min at 30 °C untreated or treated with a mixture of calpain (5 units/ml), 200 μM CaCl2, and either 200 μM calpain-

Actuated Bid (cBid, Calbiochem) or 5 mM atractyloside (Atr, Calbiochem), then centrifuged for 30 min at 13,400 × g to collect supernatants. Released proteins were separated on 4–15% SDS gels, and AIF was detected by Western blotting as described above.

AIF-DNA Interaction—Interaction of AIFΔ1–101 with linearized double-stranded DNA was examined using gel-retardation assays (25). 1–100 μg of AIF was incubated with a 100-bp DNA ladder (180–250 ng, New England Biolabs or Invitrogen) for 15 min at room temperature in 20 mM Hepes, pH 7.0, in the absence and presence of 2 mM NAD(P)(H) and equimolar amounts of recombinant human CypA (Calbiochem) or heat shock protein 70 (Hsp70) and electrophoresed in 50 mM Tris acetate on 1.2–2% agarose gel containing 0.6 μg/ml ethidium bromide. The ability of AIFΔ1–101 to degrade DNA was examined as described by Cande et al. (4). 20 nM AIF was incubated with 200 ng of a pNEB plasmid (2.7 kb, New England Biolabs) with or without 20 mM CypA for 1–4 h at 37 °C. The resulting mixtures were resolved on 1.2% agarose gels and visualized with ethidium bromide.

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Interaction of AIF with NAD(P)H

TABLE 1
Properties of recombinant AIF

| Δ1–53 | Δ1–101 | Δ1–77 H453L |
|--------|--------|-------------|
| Absorption maxima (nm) | 273, 380, 452, 476 | 273, 381, 452, 477 | 273, 380, 450, 475 |
| $\epsilon_{452}$ (mM$^{-1}$ cm$^{-1}$) | 13.5 ± 0.3 | 13.2 ± 0.3 | 13.4 ± 0.2 (13.4 ± 0.3)$^a$ |
| $E_{m}$ (mV) | −342 ± 3 | −336 ± 5 | −285 ± 5 (−341 ± 5) |
| $\Delta_f^{\text{H}}$ (mV/pH unit) | 26 | 26 | 26 (26) |
| $K_{M, \text{NADH}}$ (s$^{-1}$) | 0.2 | 0.3 | 0.6 (0.2) |
| $K_{M, \text{NADPH}}$ (M$^{-1}$ s$^{-1}$) | 0.6 | 0.5 | 0.6 (0.6) |
| $K_{M, \text{NADH}}/K_{M, \text{NADPH}}$ | 1.0 | 1.0 | 1.0 (0.3) |
| $k_{\text{cat}}$ (s$^{-1}$) | 2.7 × 10$^{-3}$ | 2.3 × 10$^{-2}$ | (1.9 × 10$^{-2}$)$^b$ |
| $k_{\text{cat}}$ (s$^{-1}$) | 1.0 × 10$^{-2}$ | 1.7 × 10$^{-2}$ | 0.6 (1.2 × 10$^{-2}$) |
| $k_{\text{cat}}$ (mM$^{-1}$) | 3.7 | 2.5 | 4.3 (3.5) |
| $k_{\text{cat}}$ (mM$^{-1}$) | 1.4 × 10$^{-2}$ | 6.8 × 10$^{-3}$ | 1.4 × 10$^{-3}$ (3.4 × 10$^{-3}$) |

$^a$ Values in parentheses are for WT AIF Δ1–77.
$^b$ Dependence of $k_{\text{cat}}$ for the reaction of AIFΔ1–77 H453L reduction with NADH on AIF concentration is hyperbolic with $k_{\text{cat}}$ of 0.6 s$^{-1}$.

Another remarkable feature of AIF was resistance of its NAD(P)H-bound forms to oxidation. Normally, it takes 2 days for air oxygen to fully oxidize FAD in CTC at ambient temperatures producing spectral changes opposite to those observed during the reduction step (not shown). To speed up the oxidation process, we measured kinetics of CTC decay under pure oxygen atmosphere. This reaction was multiphasic and accelerated with time (Fig. 1, C and D) most likely due to formation of multiple redox intermediates. Importantly, complexes with NADPH had a shorter lifetime than those with NADH, wherein AIFΔ1–53 was oxidized 2-fold slower than the corresponding redox forms of AIFΔ1–101. This was the first indication that the extended N terminus assists CTC formation/stabilization.

AIF Possesses Low Electron Transferring Ability—Kinetic parameters, such as low NADH binding rates, high $K_m$ and low $K_d$ for the cofactor, as well as the low redox potential of the flavin and high stability of the NADH-bound form, suggest that AIF cannot effectively transfer reducing equivalents from NADH to other molecules. Indeed, AIF catalyzed reduction of one- and two-electron acceptors with turnover numbers several orders of magnitude lower than those of traditional electron transferring flavoenzymes (Table 2). Notably, the 2,6-dichlorophenolindophenol (DCIP) and cytochrome $c$ reduction rates did not depend on whether catalase or superoxide dismutase was present in the reaction mixture, meaning that electrons to the acceptors came directly from AIF. The rate of nitro blue tetrazolium (NBT) reduction, on the other hand, was decreased by ~30% in the presence of superoxide dismutase and thus was partially mediated by superoxide anion, which is quite typical for oxidoreductases. Owing to high stability of CTC with NADH, the NADH oxidase activity of AIFΔ1–53 was virtually undetectable, and that of AIFΔ1–101 was very weak (<0.2 min$^{-1}$). This is in contrast to refolded
TABLE 2
NADH-dependent redox activities of AIF

|                  | $K_m$ (mM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ (mM$^{-1}$ min$^{-1}$) |
|------------------|------------|------------------------|-------------------------------------|
|                  |            |                        |                                     |
| AIFΔ1–53         |            |                        |                                     |
| DCIP             | 2.0 ± 0.2  | 42 ± 421               |                                     |
| NBT              | 1.2 ± 0.1  | 53 ± 5                 | 44                                  |
| Cytochrome c     | 1.0 ± 0.1  | 46 ± 5                 | 46                                  |
| AIFΔ1–101        |            |                        |                                     |
| DCIP             | 1.4 ± 0.1  | 48 ± 634               |                                     |
| NBT              | 0.5 ± 0.1  | 61 ± 5                 | 122                                 |
| Cytochrome c     | 1.3 ± 0.2  | 30 ± 3                 | 23                                  |
| AIFΔ1–77 H453L   |            |                        |                                     |
| DCIP             | 1.3 ± 0.1  | 248 ± 20 (32 ± 3)      | 191 (19)                            |
| NBT              | 0.6 ± 0.1  | 167 ± 7 (52 ± 4)       | 278 (40)                            |
| Cytochrome c     | 0.3 ± 0.03 | 63 ± 1 (50 ± 3)        | 217 (63)                            |
| K$_{Fe(CN)}$$_2^-$ | 0.4 ± 0.1  | 111 ± 10               | 278                                 |
| NADH oxidation$^d$ | 0.14 ± 0.01 | 2.5 ± 0.2 | 18                                  |

$^a$ Dependence of $K_m$ on DCIP concentration was not hyperbolic.
$^b$ Parameters in the parentheses are for WT AIFΔ1–77.
$^c$ NBT reduction by the mutant was inhibited at NADH concentrations higher than 1.5 mM.
$^d$ Ferricyanide reductase and NADH oxidase activities of AIFΔ1–53, Δ1–77, and Δ1–101 were negligible.

FIGURE 2. Redox-dependent changes in the oligomeric state and conformation of AIF. A–C, changes in the molecular size of AIFΔ1–53 (solid line) and Δ1–101 (dashed line) were assessed by gel-filtration under aerobic conditions. Standards: 1, ferritin, 440 kDa; 2, catalase, 232 kDa; 3, albumin, 67 kDa; 4, ovalbumin, 43 kDa; and 5, chymotrypsinogen A, 25 kDa. Ligand-free AIF migrated as a 55–60 kDa monomer (A), whereas NADH- and NADPH-reduced AIF eluted as a 110–120 kDa dimer (B and C, respectively). D and E, redox-dependent changes in the secondary structure of AIF. Far UV CD spectra of AIFΔ1–53 (D) and Δ1–101 (E) in the absence (solid bold line) or presence of a 20-fold excess of NAD (dashed line), NADP (dashed line), NADH (solid line), and NADPH (dashed and dotted line). Insets are magnified views of the 205–225 nm region. NADH binding to both AIFΔ1–53 and Δ1–101 was accompanied by an increase in the β-strand and turn content and a decrease in the percentage of random coil (see details in supplemental Fig. 5S). F and G, binding of reduced PN affects susceptibility of AIFΔ1–53 to proteolysis. F, calpain attacks oxidized AIF at Gly$^{102}$ and Glu$^{116}$, producing 55- and 53-kDa fragments, respectively, and only at Gly$^{125}$ when AIF is reduced by NADPH. In contrast, only one Arg$^{176}$-Ala$^{177}$ site is accessible to trypsin in oxidized AIF and two, Arg$^{176}$-Ala$^{177}$ and Lys$^{151}$-Ser$^{151}$ sites in the reduced protein (G). The redox-sensitive Lys$^{517}$-Ser$^{518}$ site is part of the 509–559 insertion uniquely present in AIF (shown in supplemental Fig. 5S).

AIFΔ1–120 that was shown to oxidize NAD(P)H considerably faster and catalyze NAD(P)H-dependent reduction of cytochrome c and NBT exclusively via superoxide anion radicals (9). Finally, neither form of our flavoprotein could transfer electrons from NADH to superoxide anion, hydrogen peroxide, ascorbate, free radical, or dehydroascorbate, which suggests that AIF is not an antioxidant.

AIF Undergoes Dimerization and a Conformational Change upon NAD(P)H Binding—Redox properties of AIF suggest that either the flavoprotein has evolved as a low turnover electron transferase or plays another role in mitochondria where formation of stable CTC is functionally important. To test the latter possibility, we analyzed how PN binding affects molecular properties of AIF. Gel-filtration experiments showed that oxidized AIFΔ1–101 and Δ1–53 existed in solution as monomers (Fig. 2A). NAD(P)H had no effect on the elution profile (not shown), but reduction with NAD(P)H caused a 2-fold increase in the molecular size of both proteins (Fig. 2, B and C). Thus, AIF dimerization may be one of the factors stabilizing the FADH$_2$NAD(P) complex. A small monomeric peak seen in the elution profiles of the NADPH-reduced proteins (Fig. 2C) reflects partial dissociation of the lower affinity ligand, wherein the larger amount of
monomers produced by the NADPH-bound apoptotic form (10 versus 5% in mature AIF) was another sign that the N-terminal peptide is recruited in CTC formation/stabilization.

We analyzed also how PN cofactors affect the secondary structure of AIF. Far UV CD spectra of ligand-free AIF1-53 and Δ1-101 were highly similar but not identical (Fig. 2, D and E), suggesting that the N terminus of mature AIF may adopt an ordered conformation in solution. Only subtle changes in the CD spectra of both proteins were detected in the presence of oxidized cofactors. In contrast, complex formation with NAD(P)H caused significant perturbations in the 190–250 nm region. Computer analysis of the spectroscopic data identified a common trend in the NAD(P)H-induced structural changes in AIF, such as an increase in the β-strand and turn content and a decrease in the relative percentage of random coil (supplemental Fig. 3S).

Redox-linked Conformational Changes in the N-terminal and S09–S59 Peptides—Given that proteolytic processing of the AIF N terminus is a critical event leading to caspase-independent apoptosis, we examined how PN cofactors affect susceptibility of the mature recombinant flavoprotein to specific and nonspecific proteases, calpain I and trypsin, respectively. According to the N-terminal amino sequencing, calpain attacks oxidized AIF1-53 at two sites, Gly102-Leu103 and Gly119, producing 55 and 53-kDa fragments regardless of whether or not NAD(P) was present, and only at Gly102-Leu103 when AIF was reduced with NAD(P)H (Fig. 2F). Inability of calpain to access the Glu118-Gly119 site in the AIF-NAD(P)H complex was the third indication that the N-terminal peptide, previously designated as a linker connecting the membrane-spanning and catalytic fragments of AIF with no functional role (1), is actively involved in AIF-NAD(P)H association. Unlike calpain, trypsin proteolysed only one Arg126-Ala127 bond in oxidized AIF, and two bonds, Arg126-Ala127 and Lys517-Ser518, in the NAD(P)H-reduced protein (Fig. 2G). The redox-sensitive trypsinolysis site is situated in the AIF-specific S09–S59 insertion, part of which forms two short α-helices and blocks the active site in the ligand-free protein (supplemental Fig. 4S) (10, 20). Evidently, the Lys517-containing helical stretch must unfold upon reduction to become accessible to trypsin. Because the content of random coil decreases and more β-strands are formed in the reduced protein, the 50–59 peptide likely adopts a different conformation rather than becoming fully disordered when the AIF-NAD(P)H complex is formed.

Native AIF Undergoes Redox-dependent Monomer-Dimer Transition, and Its Release from Mitochondria Is Inhibited by NAD(P)(H)—To confirm that the recombinant flavoprotein is structurally and functionally similar to the native one, we investigated properties of mitochondrial AIF. As two-dimensional BN-SDS-PAGE analysis showed, native AIF exists as an equimolar mixture of monomers and dimers in the digitonin-solubilized fraction of rodent liver mitochondria that were untreated or treated with NAD(P) prior to addition of the detergent, and predominantly dimeric when the incubation media contained NAD(P)H (Fig. 3, A and B). When NADH and NBT, an electron acceptor that turns dark-blue upon reduction, were used as substrates for in-gel reductase activity assay, only high molecular weight respiratory supercomplexes but not bands corresponding to AIF were detected (Fig. 3D). Thus, similar to recombinant AIF, the native flavoprotein forms long lived dimeric complexes with reduced cofactors and possesses a low NADH oxidase activity.

Another common feature between the proteins was susceptibility to calpain. AIF released from mitochondria treated with cBid or Atr (inducers of morphological changes and the inner membrane remodeling (27)) migrated as the 55-kDa fragment of recombinant AIF (Fig. 4A) and, according to Yuste et al. (7), was also processed at Gly102-Leu103. The smaller digestion product corresponding to the 53-kDa fragment of recombinant AIF could be detected only when permeabilized mitochondria were incubated with calpain for extended periods of time. Based on these results, one can conclude that AIF in healthy or freshly permeabilized, respiring mitochondria predominantly exists as a PN-bound dimer and converts into a ligand-free monomer when reduced cofactors are consumed.

Next, we tested how addition of various PN into the respira-
FIGURE 4. Recombinant and native AIF produce similar calpain digest patterns, and release of the mitochondrial flavoprotein is inhibited by NAD(P)H. A, native and recombinant AIF are similarly digested by calpain. AIF released from mitochondria 1 h after Atr/calpain treatment (lane 1) corresponds to the 55-kDa fragment produced during calpain digest of the NAD(P)H-bound recombinant AIFΔ1–53 (lane 4). The smaller band, corresponding to the 53-kDa calpain digest product of oxidized recombinant AIFΔ1–53 (lane 5), could be detected when permeabilized mitochondria were incubated with calpain for prolonged periods of time (lanes 2 and 3–8 and 16 h, respectively). Immunodetection of AIF was carried out as described under “Experimental Procedures.” B and C, calpain-induced release of AIF from Atr- and cBid-treated mitochondria is inhibited by PN. Mitochondria were respiring for 15 min in malate/glutamate or succinate/rotenone media supplemented with 0–5 mM NAD(P)H or 0.1 mM calpeptin, an inhibitor of calpain, prior to addition of Atr/calpain or cBid/calpain mixtures. One hour later, supernatants were collected and analyzed.

FIGURE 5. Interaction of apoptogenic AIFΔ1–101 with DNA is inhibited by NAD(P)H, and the nuclease activity of the protein is not enhanced by CypA. A, AIFΔ1–101 was incubated with 250 ng of 100-bp DNA ladder (New England Biolabs) for 15 min with or without 2 mM NAD(P)H. The mixture was separated on 2% agarose gel and visualized with ethidium bromide. B, addition of equimolar amounts of CypA did not change the DNA migration pattern, wherein in the presence of Hsp70 the inhibiting effect of NADH was eliminated. A lower concentration (180 ng) and a different type of 100-kb DNA ladder (Invitrogen) were used in these experiments to detect changes in DNA retardation at lower protein concentrations. C, the nuclease activity of AIFΔ1–101 was assessed by incubating 20 μM protein with 200 ng of plasmid DNA (2.7 kb, pNEB) for 1–4 h in the absence and presence of 20 mM CypA. Lane 1, DNA ladder; lane 15, pNEB linearized with EcoRI, SC, supercoil; OC, open circular; and L, linearized forms of DNA. Only small amounts of linearized plasmid DNA and no products of DNA degradation were detected during prolonged incubation of AIF with CypA (lanes 11–14).
Interaction of AIF with NAD(P)H

Figure 6. His^{453} is crucial for AIF-NADH interaction. A, overlay of the active sites of mouse AIF (black, PDB code 1GV4 (10)) and homologous oxidoreductases, the NADH-bound reductase component of biphenyl dioxygenase from Pseudomonas sp. (dark gray, PDB code 1F3P (44)) and putidaredoxin reductase from Pseudomonas putida (light gray, PDB code 1Q1R (45)). AIF residues are labeled. A hypothetical movement of the side chain of His^{453} to establish a direct hydrogen bond with the nicotinamide and assist in orienting and tighter binding of the cofactor is shown by an arrow. B, anaerobic titration of AIF_{1–77} with NADH. K_d for NADH calculated from the plot shown in the inset was 300-fold higher than that determined for WT (40 μM versus 150 nM, respectively). C and D, CD signals produced by WT (solid line) and H453L with NADH. CD signals of correctly folded oxidized and NADH-bound AIF is necessary to perturb AIF-NADH interaction and stability of CTC, we conclude that notable changes in DNA retardation were caused by formation of a specific complex between Hsp70 and reduced AIF. Another important observation was that our AIFΔ1–101 displayed negligible DNA nicking and linearizing abilities and, unlike the refolded flavoprotein (4), could not degrade supercoiled plasmid DNA in the presence of CypA (Fig. 5C). This suggests that in the nucleus of a dying cell AIF may recruit proteins other than CypA to effectively initiate chromatin degradation or, as Yuste et al. found (32), may not be involved in DNA fragmentation at all.

The Active Site His^{453} Is Crucial for NADH Binding—To get an insight into how CTC is stabilized and how redox-linked changes in the active site are transmitted to the surface, we compared crystal structures of AIF and other oxidoreductases of the glutathione reductase family. As seen from a structural overlay (Fig. 6A), the active sites are highly conserved except for one distinct residue in AIF, His^{453}, substituted by Leu or Ile in other homologues. The H453L replacement was found to profoundly affect redox properties of AIF. Compared with WT, the mutant had a 56-mV higher redox potential (Table 1), two orders of magnitude lower affinity for NADH (K_d of 40 μM), more effectively catalyzed electron transfer reactions (Table 2) and reacted with the cofactor without CTC formation (Fig. 6B), producing a structurally different and easily oxidizable intermediate (Fig. 6C–F). Hence, His^{453} is a key residue that not only modulates the FAD redox potential but also defines the manner of AIF-NADH interaction. Being situated in the vicinity of the isoalloxazine ring of FAD, the imidazole ring of His^{453} could directly interact and optimally orient the nicotinamide group for CTC formation, wherein its positional shift, shown by an arrow in Fig. 6A, could transmit the redox signal to the surface via adjacent peptides. Given that, similar to the H453L mutant, refolded AIF does not form CTC with equimolar NAD(P)H (9), it is plausible to suggest that the His^{453}-containing peptide is misplaced during refolding. Evidently, determination of three-dimensional structures of correctly folded oxidized and NADH-bound AIF is necessary to precisely elucidate the redox mechanism and identify alterations caused by refolding.
Interaction of AIF with NAD(P)H

There are two important conclusions that follow from this work. First, our finding that the manner of FAD incorporation and the length of the N terminus define redox properties of AIF implies that previous studies involving truncated, refolded, and tag-fused fragments of the protein need to be carefully reexamined, whereas future investigations must ensure that correctly folded AIF is employed. Secondly and most importantly, our results provide novel insights into the redox mechanism of AIF suggesting that, in addition to catalyzing electron transfer reactions, the flavoprotein may play a role in redox signaling.

Protein monomer-oligomer transition is a key process in cellular signal transduction. The AIF-NAD(P)H complex formation triggers not only dimerization but also a conformational change in the 509–559 peptide and other, yet to be identified parts of the molecule. We propose that these redox-linked structural changes are functionally important. The 509–559 stretch of amino acids contains two regulatory elements (supplemental Fig. 5S): the proline-rich motif (PPXXPXXPXXP), a putative recognition/interaction site for Src homology 3 domains found in numerous cytoskeletal and signaling proteins (33) and capable of mediating assembly of large multiprotein complexes (34), and the proline/glutamate-serine/threonine-rich (PEST) sequence, a potential proteolytic signal and phosphorylation site. Some of the PEST sequences, present in key metabolic enzymes that exhibit rapid changes in concentration and must be short-lived (35), are conditional proteolytic signals masked in one form of the protein and activated in another via conformational reorganization or serine/threonine phosphorylation. Given that the conformation and solvent exposure of the 509–559 peptide are defined by the redox state of FAD, it is plausible to hypothesize that the AIF-specific insertion represents a regulatory element through which redox changes in the active site are transmitted to the surface and further to the neighboring/interacting proteins. This way, NAD(P)H binding could control AIF-mediated signaling pathways as well as the lifetime, nuclear translocation of the flavoprotein, and, as demonstrated here, its interaction with DNA.

Mutagenesis studies on human AIFΔ1–120 help to understand how ligand association and a conformational switch in the regulatory peptide could change affinity for DNA. Ye and coworkers (20) showed that replacement of Lys510 and Lys518 (Lys509 and Lys517 in mouse AIF, respectively) with Ala prevented DNA binding and induction of cell death. Being spatially oriented in oxidized AIF (supplemental Fig. 5S), these lysines could contribute to a positive electrostatic surface potential and promote interaction with the negatively charged DNA. Elimination of positive charges or, as this study predicts, NAD(P)H-
induced AIF dimerization and a conformational switch in the 509–559 peptide could change the surface charge distribution and lower affinity for DNA.

Our results suggest also that the redox-induced structural reorganization may affect interaction of AIF with Hsp70 (Fig. 5B), a cryoprotectant that inhibits apoptosis through association and retaining AIF in the cytosol (29–31). When cellular levels of NAD(P)H are sufficiently high, a preferable sequestering of reduced AIF by Hsp70 would be beneficial, because it could slow down or prevent initiation of an AIF-mediated cascade of apoptotic events and give the damaged cell a chance to recover. And vice versa, depletion of reduced PN leading to AIF oxidation and monomerization would manifest serious aberrations in the cellular homeostasis and retaining of the flavoprotein in the cytoplasm would be of little help. Although we did not obtain any evidence for cooperation between AIF and CypA in DNA binding and degradation, a possibility of their direct interaction exists and needs to be further explored. Nucleocytoplasmic transport can be regulated by post-translational modification (phosphorylation, methylation, and ubiquitination) of target proteins (36). Given that the 509–559 loop is a putative phosphorylation site and AIF was shown to undergo serine and threonine phosphorylation (9) and ubiquitination (37), it is possible that interaction of the flavoprotein with CypA as well as other partners, such as T-cell ubiquitin ligand and X-linked inhibitor of apoptosis (37, 38), is controlled by the redox state-dependent modification of the flavoprotein.

In contrast to our results, several in vivo and in vitro studies led to a conclusion that the apoptogenic function of AIF does not depend on its oxidoreductase activity (1, 2, 9, 39). As a control in apoptotic assays, these studies employed refolded, polyhistidine-tagged recombinant or intracellularly expressed AIF fused to the green fluorescent protein. Because truncation, misfolding, and attachment of charged or bulky markers could perturb not only the FAD redox potential, NAD(P)H binding, and dimerization of AIF but also its interaction with DNA and other proteins, there is a possibility that the PN-binding ability of the control proteins was perturbed and, as a consequence, assessment of the redox dependence of the apoptogenic action of AIF was inaccurate. Another observation used as an argument is that AIFsh, a naturally occurring isoform consisting of the C-terminal residues 353–613, provokes the same apoptotic effect as AIFΔ1–120 (21). The structure of the 353–613 fragment derived from the x-ray model of AIFΔ1–120 (supplemental Fig. 6S) suggests that AIFsh must aggregate or adopt a different conformation in solution to stabilize long surface loops and bury hydrophobic patches. If the structures of AIFsh and AIFΔ1–120 differ, their apoptotic mechanisms may differ as well.

Finally, for the first time, we showed that processing/release of mitochondrial AIF can be regulated by PN cofactors. Little is known about PN homeostasis in the IMS, and elucidation of the mechanism underlying our findings requires special investigations. Nevertheless, based on the results presented here and reported previously (12–17, 19) as well as the facts that massive depletion of cellular NAD(P)H is an early event in apoptosis (40), mitochondria are the major store of NAD that can leak into or be replenished from the cytosol (41, 42), and the redox state of the NAD(P)(H) pool is an important modulator of mitochondrial function (43), we hypothesize that mitochondrial AIF may act as a redox sensor whose normal and apoptogenic functions are controlled by NAD(P)H according to the following mechanism (Fig. 7). Under normal conditions, when reduced cofactors are abundant in mitochondria, AIF predominantly exists as a NAD(P)H-bound dimer and participates in redox cycling, cristae formation, and proper assembly/functioning of the respiratory chain directly or indirectly via protein–protein interactions. PN depletion and aberrations in the inner membrane can transiently disrupt multiprotein complexes and increase reactive oxygen species production (reversible, pre-apoptotic state). Prolonged PN deficiency and morphological changes coupled with protease activation and increased permeability of the outer membrane lead to AIF proteolysis and release from the IMS (point of no return). Ligand-free monomers translocate to the nucleus and initiate caspase-independent apoptosis, wherein PN-bound dimers may be retained in the cytosol. If NAD(P)H-bound AIF does enter the nucleus, its apoptogenic ability would be considerably lower due to the lower affinity to DNA. Elucidation of whether AIF can function via the outlined mechanism and link PN-dependent metabolic pathways to apoptosis is vital for better understanding of cellular communications and developing new therapeutic strategies and should be the focus of future studies.

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