DNA Binding Suppresses Human AIF-M2 Activity and Provides a Connection between Redox Chemistry, Reactive Oxygen Species, and Apoptosis

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Human AIF-M2 is an unusual flavoprotein oxidoreductase that binds DNA, nicotinamide coenzyme, and the modified flavin 6-hydroxy-FAD. Using multiple solution methods to investigate the redox chemistry and binding interactions of AIF-M2, we demonstrate that binding of DNA and coenzyme to AIF-M2 is mutually exclusive. We also show that DNA binding does not perturb the redox chemistry of AIF-M2, but it has significant effects on the reduction kinetics of the 6-hydroxy-FAD cofactor by NAD(P)H. Based on quantitative analysis of ligand binding and redox chemistry, we propose a model for the function of AIF-M2. In this model, DNA binding suppresses the redox activity of AIF-M2 by preventing the binding of the reducing coenzyme NAD(P)H. This DNA-mediated suppression of AIF-M2 activity is expected to lower cellular levels of superoxide and peroxide, thereby lessening survival signaling by Ras, NF-κB, or AP-1, as suggested from knock-out studies of the related AIF in human colon cancer cell lines. We show marked differences between AIF-M2 and AIF. DNA and coenzyme binding activity is retained in the C-terminal deletion mutant AIF-M2-(A319 – 613), whereas DNA binds to the C-terminal D3 domain of AIF. Our work provides the first analysis of AIF-M2 ligand interactions and redox chemistry and identifies an important mechanistic connection between coenzyme and DNA binding, redox activity, and the apoptotic function of AIF-M2. Through its DNA binding activity, we suggest that AIF-M2 lessens survival cell signaling in the presence of foreign (e.g. bacterial and (retro)viral) cytosolic DNA, thus contributing to the onset of apoptosis.

Apoptosis (programmed cell death), a cellular process in higher organisms by which cells die (1, 2), can be induced by toxic insult (e.g. chemical damage) or physical disruption of cells. It is critical in the development of multicellular organisms, including the development of the embryo and vital organs, and in cellular homeostasis (3). Up to 70 billion human adult cells undergo apoptosis each day (4). Lesions in the apoptotic process or its regulation result in developmental defects, immortalized cells, and cancers (3). The mitochondrion is a key apoptosis regulator, and pro-apoptotic and cell damage-controlled pathways feed back to the mitochondrion and induce permeabilization of mitochondrial membranes (5). In part, this is under the control of the Bcl-2 protein family, which contains members with either pro- (e.g. Bax) or anti-apoptotic (e.g. Bcl-2) functions (6). Cytotoxic molecules in the mitochondrial intermembrane space are released on membrane permeabilization and act as cell death effectors, some depending on activation by caspase proteases (7). Release of cytochrome c triggers activation of the initiator caspase-9. Downstream activation of other caspases leads to proteolytic cleavage of key target molecules (e.g. laminins) leading to cell morphology changes such as plasma membrane perturbations, condensation and fragmentation of nuclear chromatin, and compaction of cytoplasmic organelles and cell volume (8). Caspase-independent pathways of cell death are also known, initiated by molecules released from mitochondria such as endonuclease G and the flavoprotein apoptosis-inducing factor (AIF) (9).

AIF is a FAD- and NAD(H)-binding enzyme with a glutathione reductase-like fold (10, 11). AIF is confined to the mitochondrion and released in response to cell death signaling, likely mediated by the enzyme PARP-1, a nuclear enzyme, activated by DNA damage (12). Translocation of mature AIF (i.e. lacking its mitochondrial targeting sequence) to the nucleus induces apoptosis by fragmentation of high molecular weight DNA and chromatin condensation (13). AIF is a DNA-binding NADH-dependent oxidoreductase (13), comprising three major domains (D1, D2, and D3). D1 and D2 bind FAD and NAD(H). The C-terminal D3 domain is involved in DNA binding important to the physiological role(s) of AIF (10, 11). AIF is a critical early nuclear effector of apoptosis, but key aspects of its cellular functions are unresolved. AIF is monomeric in solution but crystallizes as a dimer (10). It has no nuclelease activity (14) but may recruit other protein factors (e.g. nucleases) that enable chromatinolysis and apoptosis (11). It has been suggested the AIF redox FAD-binding domain is dispensable for apoptotic activity (15). For instance, mice suffering from a neu-

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S6, Table S1, and additional references.

2 The abbreviations used are: AIF, apoptosis-inducing factor; ROS, reactive oxygen species; TEV, tobacco etch virus; dsDNA, double-stranded DNA; 2-AP, 2-aminopurine.
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rododegenerative disorder showed a prorval insertion in the AIF gene resulting in severe AIF depletion and increased susceptibility to peroxide-induced apoptosis (16). However, knocking out AIF activity in different human colon cancer cell lines leads to loss of tumorigenicity, enhanced apoptosis sensitivity, and lower superoxide levels (17). This suggests lessened survival signaling mediated by Ras, NF-κB, or AP-1 (17). The precise mechanistic links between redox activity and cell survival are unknown.

In humans, an ortholog of AIF was recognized recently (18, 19). The flavoprotein AMID (AIF-homologous mitochondrion-associated inducer of death), now referred to as AIF-M2, has sequence similarities to AIF but apparent functional differences. AIF-M2 has a pro-apoptotic function and was initially recognized as a p53 target gene whose expression was induced in human colon cancer cells undergoing p53-dependent apoptosis (18, 21–22). Expression is down-regulated in cancer cell lines by comparison with non-tumor cell lines from the same tissues (21, 22). Overexpression of AIF-M2 in human embryonic kidney cells, HeLa cells, and other cell lines induces apoptosis. Expression of AIF-M2 induces cellular apoptosis at much lower levels than AIF in a number of different cell lines (18, 21). Unlike AIF, AIF-M2 is predominantly cytosolic and may have affinity for the cytosolic surface of the mitochondrial outer membrane. This is consistent with the apparent lack of an extended N-terminal mitochondrial targeting sequence analogous to that found in AIF (18, 21). AIF-M2 is transcriptionally up-regulated by p53 and by genotoxic chemicals, suggesting that it might contribute to the arrest of cell growth on exposure to such agents (23). AIF-M2 is a DNA-binding protein with nicotinamide coenzyme-dependent oxidoreductase activity, and it is usual for the purified protein to contain 6-hydroxy-FAD (19).

Here we have performed detailed analysis of the DNA- and coenzyme-binding properties of AIF-M2 and investigated effects on redox chemistry using equilibrium and fast reaction methods. The analysis has enabled us to establish a link between DNA-binding properties and redox activity of AIF-M2 that likely affects the levels of reactive oxygen species (ROS), survival signaling, and apoptosis. We suggest that regulation of AIFM-2 activity through DNA binding, which contributes to the onset of apoptosis, is important when foreign cytosolic DNA is present (e.g. during infection).

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Site-directed mutagenesis of plasmid pET15b AIF-M2 (19) was performed to exchange the thrombin recognition site for a TEV protease recognition sequence (ENLYFQG) using the following mutagenic primers: 5′-GGA CCC CAT ATG GCC TGT GGA ATA CAA ATT TTC CCT GCT GTG ATG ATG ATG TAG ATG GC-3′ and 5′-GCC ATC ATC ATC ATC ATC ATC ACA GCA GGA AAA ATT TGT ATT TCC AAG GCC ATA TGG GTG GGT CC-3′. Following mutagenesis, the entire gene was re-sequenced to ensure spurious changes had not occurred during the mutagenesis procedure. AIF-M2 protein was expressed in Escherichia coli strain HMS174 (DE3) and purified as described previously (19). TEV protease was used to remove the N-terminal histidine tag. Cleavage was performed in 50 mM potassium phosphate buffer, pH 8.0, containing 100 mM KCl, 0.5 mM EDTA, and 1 mM dithiothreitol for 12 h at 4 °C. The ratio of AIF-M2 to TEV protease was 100:1. Following digestion with TEV protease, the protein was subjected to gel filtration (S-200 column) to remove EDTA. TEV protease, which is His-tagged, was subsequently removed from the AIF-M2 preparation by chromatography using a nickel-nitrilotriacetic acid resin. AIF-M2 was eluted from this resin in 50 mM potassium phosphate, pH 8.0, containing 100 mM potassium chloride and 10 mM imidazole. Imidazole was subsequently removed from purified AIF-M2 using a DG-10 desalting column. AIF-M2 was stored in 50 mM potassium phosphate buffer, pH 8.0, containing 100 mM potassium chloride and 10% glycerol at −20 °C.

DNA Binding Assays—Gel retardation assays were used to examine the interaction of AIF-M2 with DNA and to investigate the effect of NADP+ on DNA binding. These assays used the following oligonucleotides: 5′-ACT GAC TGA CTG ACT GAC TGA CTG A and 5′-TCA GTC AGT CAG TCA GTC AGT CAG T-3′, which were annealed to form a 25-bp double-stranded DNA (dsDNA). AIF-M2 was incubated with dsDNA for 20 min at room temperature, in the presence or absence of pyridine nucleotide coenzyme, in 50 mM potassium phosphate buffer, pH 8.0, 100 mM potassium chloride. Samples were then electrophoresed in 1.5% agarose gels. DNA was visualized by staining with ethidium bromide.

Quantitative analysis of DNA binding to AIF-M2 was achieved using a fluorescence binding assay employing the fluorescent dsDNA analog comprising the following annealed oligonucleotides: 5′-ACT GAC TGA CTG ACT GAC TGA CTG A and 5′-TCA GTC AGT CAG TCA GTC AGT CAG T-3′; where a represents 2-aminopurine (2-AP) (a fluorescent analog of adenine (24)). Fluorescence assays were performed using a Cary Eclipse fluorescence spectrometer (Varian) and a 10-mm Hellma fluorescence cuvette. Excitation wavelength was 310 nm, and fluorescence emission was monitored at 370 nm. Binding assays were conducted in 50 mM potassium phosphate buffer, pH 8.0, containing 100 mM potassium chloride. Titrations were performed with 10 μM 2-AP DNA in the cuvette. AIF-M2 was titrated against 2-AP DNA (AIF-M2 concentration range 0–100 μM), and fluorescence changes were corrected for dilution effects. Control titrations were performed by adding buffer (50 mM potassium phosphate buffer, pH 8.0, containing 100 mM potassium chloride) or protein (bovine serum albumin) to 2-AP DNA. Titrations were also performed in the presence of NADP+ (see “Results”). The change in fluorescence emission was plotted against AIF-M2 concentration, and the apparent dissociation constant for the AIF-M2-2-AP DNA complex was determined by fitting to Equation 1.

$$
\Delta F = \Delta F_{\text{max}} \left( \frac{(L_R + E_T + K_d) - ((L_R + E_T + K_d)^2 - (4L_R E_T))^{1/2}}{2E_T} \right)
$$

(Eq. 1)

where $L_R$ is total ligand concentration; $E_T$ is total enzyme concentration; $\Delta F_{\text{max}}$ is the maximum change in fluorescence emission; and $K_d$ is the dissociation constant for the AIF-M2-2-AP DNA complex.
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Construction of a C-terminally Deleted Form of AIF-M2—A C-terminal deletion of the AIF-M2 protein was constructed by mutagenesis using the Stratagene QuickChange procedure using the following oligonucleotides: 5′-CTC CAG GCC TAG TAG CCG GGT GCA CTG ACG-3′ (forward primer) and 5′-CGT CAG TGC ACC CGG CTA CTA GGC CTG GAG-3′ (reverse primer). The underlined bases indicate the position of translational termination codons engineered after residue Leu-319. The truncated form of AIF-M2 was expressed from plasmid pET15b, and protein expression and purification were as described for the wild-type AIF-M2 protein (see above).

Stopped-flow Kinetic Analyses—Rate constants for the binding of 2AP-DNA to AIF-M2 were determined using fluorescence stopped-flow approaches using an Applied Photophysics SX.18MV reaction analyzer. The excitation wavelength was 310 nm and monochromator slit width 1 mm; a band pass (10 nm, centered on 370 nm) filter (7006-155, Davin Photonics) was used in the analysis of 2-AP DNA fluorescence emission. Reactions were performed in 50 mM potassium phosphate buffer, pH 8.0, containing 100 mM potassium chloride.

Binding transients were obtained for AIF-M2 (25 μM, reaction cell concentration) mixed with 2-AP DNA (concentration range 0–100 μM). Fluorescence transients were analyzed by fitting to a single exponential expression and observed rates calculated using Spectrakinetics software (Applied Photophysics). The rate of dissociation of the AIF-M2-2-AP DNA complex induced by NADP+ binding was examined using the same stopped-flow method. AIF-M2 (25 μM, reaction cell concentration) and 2-AP DNA (5 μM, reaction cell concentration) were incubated in one syringe of the stopped-flow apparatus and then mixed rapidly with NADP+ (reaction cell concentration range 0–5 mM).

Reduction of the enzyme-bound 6-hydroxy-FAD cofactor by NAD(P)H was monitored using stopped-flow absorption spectroscopy. Reductive transients were analyzed for AIF-M2 alone and for the AIF-M2-DNA complex at 25 °C in 50 mM potassium phosphate buffer, pH 8.0, 100 mM potassium chloride under anaerobic conditions. The stopped-flow instrument was contained in a Belle Technology anaerobic chamber (<5 ppm oxygen). Buffers were degassed and made anaerobic by purging with nitrogen gas for 40 min. Solutions of AIF-M2 were made anaerobic by gel filtration through a Bio-Rad DG-10 column contained in the glove box. AIF-M2 (10 μM, reaction cell concentration) was mixed with either NADH (reaction cell concentration range 0–0.5 mM) or NADPH (reaction cell concentration range 0–0.5 mM), and reduction of the flavin was monitored at the cofactor absorption maximum (430 nm). Reaction transients were biphasic, and observed reaction rates were obtained by fitting to a standard double exponential expression using Spectrakinetics software (Applied Photophysics).

Spectral changes occurring during reduction of AIF-M2 (15 μM reaction cell concentration) or the AIF-M2-DNA complex (15 μM reaction cell concentration) with either NADH or NADPH (reaction cell concentration range 30–150 μM) were analyzed using photodiode array spectroscopy. Data were collected using X-Scan software (Applied Photophysics Ltd.). Spectra were analyzed globally using the Applied Photophysics Pro-K software package. The rate constants for re-oxidation of NADPH-reduced AIF-M2 and the AIF-M2-DNA complex by molecular oxygen were analyzed by stopped-flow absorption spectroscopy. AIF-M2 (20 μM syringe concentration) or the protein DNA-complex (20 μM syringe concentration) was reduced by 17 μM NADPH in 50 mM potassium phosphate buffer, pH 8.0, 100 mM potassium chloride under anaerobic conditions. Different oxygen concentrations contained in the same reaction buffer were made by mixing anaerobic buffer with aerobic buffer prior to mixing with the NADPH-reduced protein samples. Re-oxidation of 6-hydroxy-FAD cofactor was monitored at 430 nm. Absorption transients were monophasic, and rate constants were obtained by fitting to a single exponential function.

Spectroelectrochemical Redox Potentiometry—The midpoint redox potentials of the 6-hydroxy-FAD cofactor in AIF-M2 (and in the AIF-M2-DNA complex and the AIF-M2-NADP+ complex) were examined by spectroelectrochemical titration. Redox titrations were performed in a Belle Technology glove box under a nitrogen atmosphere. All buffers and solutions were degassed by bubbling with argon prior to entry into the glove box to ensure removal of all traces of dioxygen. The protein was applied to a Bio-Rad DG-10 desalting column in the anaerobic box, pre-equilibrated with degassed 50 mM potassium phosphate buffer, pH 8.0, containing 100 mM potassium chloride and 10% (v/v) glycerol (titration buffer), to ensure removal of all traces of oxygen. The protein solutions were titrated electrochemically according to the method of Dutton (25) using sodium dithionite as reductant. Dithionite was delivered in ~2-μl aliquots from concentrated stock solutions (typically 10–50 mM). Mediators were added to facilitate electrical communication between enzyme and electrode, prior to titration. 2 μM phenazine methosulfate, 5 μM 2-hydroxy-1,4-naphthoquinone, 0.5 μM methyl viologen, and 1 μM benzyl viologen were included to mediate in the range between +100 and −480 mV, as described previously (26). The electrode was allowed to stabilize between each addition. Spectra (300–800 nm) were recorded using a Cary UV-50 Bio UV-visible scanning spectrophotometer. The electrochemical potential of the solution was measured using a Hanna pH 211 meter coupled to a Pt/Calomel electrode (ThermoRussell Ltd.) at 25 ± 2 °C. The electrode was calibrated using the Fe3+/Fe2+ EDTA couple as a standard (+108 mV). A factor of +244 mV was used to correct relative to the standard hydrogen electrode. Data manipulation and analysis were performed using Origin software (OriginLab, Northampton, MA). Absorbance values at 430 nm (near the absorption maximum for the oxidized flavins) were plotted against the applied potential. Data were fitted using Equation 2, which represents a concerted 2-electron redox process derived by extension to the Nernst equation and the Beer-Lambert Law,

\[
A_{430} = A_O + (A_R - A_O) \frac{10^{E_1 - E_2/(R/T)}}{1 + 10^{E_1 - E_2/(R/T)}}, \quad 1 \leq n \leq 2
\]

(Eq. 2)

where \(A_{430}\) is the absorbance value at 430 nm at the electrode potential \(E\), and \(A_O\) and \(A_R\) are the absorbance values of the fully oxidized and reduced enzyme, respectively, at 430 nm, and \(n\) is
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FIGURE 1. Analysis of DNA binding to AIF-M2 by gel retardation assay. A, effects of AIF-M2 concentration on the migration properties of the double-stranded oligonucleotide. Conditions are as follows. The dsDNA sample (25 bp, 5 μM) was incubated with AIF-M2 (5 μM) for 20 min at room temperature prior to loading on the gel. AIF-M2 concentration was 0, 5, 10, 20, 50, and 100 μM (lanes 1–7, respectively; lane 8, same as for lane 1, B, apparent antagonistic effects of NADP⁺ on oligonucleotide binding to AIF-M2. AIF-M2 (75 μM) was incubated with 5 μM DNA for 20 min at room temperature. After this time, NADP⁺ was added to the sample prior to electrophoresis in the agarose gel. NADP⁺ concentration was 10, 20, 50, 100, 200, and 500 μM (lanes 1–8, respectively). Incubation buffer was 50 mM potassium phosphate, pH 8.0, containing 100 mM potassium chloride.

FIGURE 2. Absorption changes accompanying the binding of NADP⁺ to AIF-M2 in the absence (A) and presence (B) of DNA. A, plot of absorption change at 430 nm on titrating AIF-M2 (10 μM) with NADP⁺. The data were fitted to a quadratic binding equation with $K_d = 13 ± 5 \mu M$ and $[AIF-M2] = 11 ± 3 \mu M$. Inset, direction of absorption changes of the AIF-M2 protein on adding NADP⁺ ligand. B, plot of absorption change at 430 nm on titrating AIF-M2 (10 μM) in the presence of DNA (30 μM) with NADP⁺. The data were fitted to a form of Equation 1 (solid line) with $K_d^{DNA} < 1 \mu M$ when $K_d^{NADP⁺} = 13 \mu M$ (fixed). Conditions are as follows. Binding assays were performed in 50 mM potassium phosphate buffer, pH 8.0, containing 100 mM potassium chloride at 25 °C.

The apparent number of electrons transferred during the reaction. By using Equation 2 to fit the absorbance potential data, the variables were unconstrained, and regression analysis provided values in close agreement to those of the initial estimates. Throughout the titration the enzyme remained soluble, and corrections for turbidity were not required.

Materials—Plasmid pET15b harboring TEV protease that contains an N-terminal histidine tag was the kind gift of Dr. S. Fullerton (University of Leicester, Leicester, UK). TEV protease was purified essentially as described for the AIF-M2 protein (above) following growth of E. coli HMS174 (DE3) on TB media containing kanamycin (50 μg/ml).

RESULTS

Binding of DNA to AIF-M2—Previously, we showed qualitatively that AIF-M2 has nonsequence specific DNA binding activity and that this binding was affected by the presence of pyridine nucleotides (19). Here we have extended these studies to provide quantitative information on DNA binding to AIF-M2 and to demonstrate the antagonistic effects of pyridine nucleotide coenzyme binding, prior to performing detailed analysis of the functional properties of AIF-M2 alone and of the AIF-M2-DNA complex. We have synthesized a self-annealing 25-bp oligonucleotide sequence for quantitative binding studies. The single-stranded version of this DNA sequence does not bind to AIF-M2, as shown by gel retardation analysis. When annealed through heat treatment and subsequent cooling, however, the double-stranded oligonucleotide binds to AIF-M2 and results in retardation of migration of the DNA in a gel retardation assay (Fig. 1A). Moreover, we have demonstrated that the binding of NADP⁺ releases DNA from the AIF-M2-DNA complex (Fig. 1B). The gel retardation analysis serves to illustrate the suitability of the DNA substrate for the quantitative binding studies reported below.

Direct Determination of the Dissociation Constant for the AIF-M2-NADP⁺ Complex by Absorption Spectroscopy and

Effects of DNA Binding on Coenzyme Binding and AIF-M2 Reduction—The dissociation constant for the AIF-M2-NADP⁺ complex was determined directly by performing absorption analysis of AIF-M2 protein titrated with NADP⁺ (Fig. 2A). Absorption changes are apparent around 430 nm on titrating AIF-M2 (10 μM) with NADP⁺, and analysis of this absorption change as a function of NADP⁺ with a quadratic binding equation (Equation 1) yielded a dissociation constant for the complex of $13 ± 5 \mu M$ and a stoichiometry of 1.1 ± 0.3. Binding of NADP⁺ to the pre-assembled AIF-M2-DNA complex also gave rise to similar absorption changes around 430 nm (Fig. 2B). As this reaction involves multiple steps with substrates, these data were analyzed as follows. The coenzyme (N)-induced dissociation of DNA from AIF-M2 (E) can be described according to Scheme 1.

$$E \cdot DNA \xrightleftharpoons{K_d^{DNA}} E + DNA + N \xrightarrow{k_i} E \cdot N \xrightarrow{k_i} E' \cdot N$$

**SCHEME 1**
The final step in parentheses describes the situation either when AIF-M2 is reduced (when \( N = \text{NAD(P)H} \)) or a second conformational change on binding nonreducing coenzymes such as NADP\(^+\) (see below). The rate constant, \( k_{\text{obs}} \), for either AIF-M2 reduction or NADP\(^+\) binding to the DNA-bound enzyme is described by Equation 3.

\[
k_{\text{obs}} = k_r + k_2 [E \cdot N] / [E]_0
\]

**(Eq. 3)**

In Scheme 1, the fraction of \( E \cdot N \) can be described by a weak-binding isotherm (27), which simplifies to the more common concentration dependence when \([\text{DNA}] = 0\) or when \( K_d^\text{DNA} > [\text{DNA}] \) (i.e. when no DNA is bound). For the data shown in Fig. 2B, when \( K_d^\text{NADP} = 13 \) \( \mu M \) (Fig. 2A), analysis using Equation 4 indicates that \( K_d^\text{DNA} < 1 \) \( \mu M \).

\[
[E + N] = [N] + K_d^0 (1 + [DNA]/K_d^\text{DNA})
\]

**(Eq. 4)**

It follows that NADP\(^+\) will bind to AIF-M2 in the presence of DNA if \( K_d^\text{NADP} \times [\text{NADP}^+] > K_d^\text{DNA} \times [\text{DNA}] \) as was the case in Fig. 2B. To obtain a more accurate \( K_d^\text{DNA} \) value, we examined directly DNA binding to the enzyme (see below).

**Quantitative Analysis of DNA Binding to AIF-M2**—Quantitative analysis of DNA binding to AIF-M2 was performed using a fluorescent analog of the double-stranded oligonucleotide in which three adenine bases were substituted for 2-aminopurine. Titration of AIF-M2 leads to a quenching in fluorescence emission at 370 nm. This quenching of fluorescence saturates at AIF-M2 concentrations around 30 \( \mu M \) (Fig. 3A). Analysis of the data using a quadratic binding equation (Equation 1) indicates a dissociation constant, \( K_d = 0.9 \pm 0.2 \) \( \mu M \) and \([\text{AIF-M2}] = 2.1 \pm 0.5 \) \( \mu M \). The apparent enzyme concentration is only \( \sim 20\% \) of the total used in the binding assay. Assuming there is a 1:1 AIF-M2:DNA ratio upon binding, this suggests that \( \sim 80\% \) of the purified protein is already bound to DNA. This notion is supported by dynamic light scattering data on purified AIF-M2 (supplemental Figs. S1 and S2). That most of the purified protein is isolated with DNA bound supports the view that this activity has physiological relevance as this DNA must have been bound in vivo.

Addition of NADP\(^+\) to the pre-formed AIF-M2-DNA complex leads to an increase in the fluorescence emission of 2-AP DNA, indicating that NADP\(^+\) displaces the DNA from the AIF-M2-DNA complex (Fig. 3B). Analysis of these data using Equation 4 yielded \( K_d^\text{DNA} = 0.12 \pm 0.05 \) \( \mu M \) when \( K_d^\text{NADP} \) is fixed to 13 and 18 \( \mu M \) (see Fig. 2A).

**C-terminal Deletion Mutant of AIF-M2 and Effects on DNA Binding and Cofactor Composition**—Studies with AIF have indicated that the C-terminal D3 domain contains a long insertion, absent from related oxidoreductases (e.g. mitochondrial adrenodoxin reductase), that is likely involved in DNA binding (supplemental Fig. S3). Given the importance of DNA binding to the physiological role(s) of AIF and AIF-M2, we created a C-terminal deletion mutant of AIF-M2 to assess if this region of the protein is involved in DNA binding.

The deletion mutant of AIF-M2 was purified using the same procedure described for wild-type AIF-M2. The pure mutant enzyme had a UV-visible spectrum typical of a FAD-containing protein, and not 6-hydroxy-FAD as for wild-type protein (Fig. 4, inset). Reduction with \( \text{NAD(P)H} \) produced the 2-electron reduced (dihydrolflavin) form of the protein. The absorbance at long wavelength (>550 nm) indicates the formation of an \( \text{FADH}_2/N\text{AD(P)}^+ \) charge-transfer complex with both \( \text{NADH} \) and \( \text{NADPH} \) (Fig. 4, inset). Re-oxidation of this charge-transfer species by molecular oxygen regenerated the oxidized (FAD) form of the enzyme and not the 6-hydroxy-FAD form as seen for the FAD-reconstituted wild-type form of AIF-M2 (19).

Quantitative analysis of DNA binding with 2-AP DNA indicated that the mutant AIF-M2 protein retained an ability to bind dsDNA (\( K_d = 0.63 \pm 0.28 \) \( \mu M \)) with affinity similar to that observed for the wild-type protein. For wild-type AIF-M2, the apparent enzyme concentration determined by fitting to a quadratic binding equation is only \( \sim 40\% \) of the total enzyme concentration, suggesting that \( \sim 60\% \) of the purified mutant protein is bound to DNA. That the \( K_d^\text{DNA} \) values for wild-type and truncated AIF-M2 are similar indicates that, unlike for AIF (10,
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Stopped-flow Analysis of the Binding of DNA and NADP\(^+\) to AIF-M2—Stopped-flow fluorescence measurements were used to analyze DNA and NADP\(^+\) binding to AIF-M2. Rapid mixing of AIF-M2 with 2-AP DNA showed a rapid quench in fluorescence of the double-stranded DNA consistent with the DNA binding seen in Fig. 3. Typical transients for this binding reaction are found in supplemental Fig. S4A. The binding of DNA to AIF-M2 was described by a simple second-order reaction (where \(k_{\text{obs}} = k_f [\text{DNA}] + k_r\) with \(k_f = 5.4 \pm 0.2 \times 10^4 \text{ M}^{-1} \text{s}^{-1}\) and \(k_r = 78 \pm 1 \text{ s}^{-1}\) (Fig. 5, inset)). The rate of DNA displacement from the AIF-M2-DNA complex, \(k_r\), is relatively fast suggesting that this step is unlikely to be rate-limiting during displacement of the DNA with nucleotide coenzymes. These displacement reactions were also analyzed by rapid mixing of NADP\(^+\) with a solution of AIF-M2 pre-mixed with 2-AP DNA. In this case, coenzyme displaces DNA from the protein with concomitant increase in fluorescence emission. Reaction transients were monophasic (supplemental Fig. S4B) and fitted using a single exponential expression. A plot of the observed rate constants for the fluorescence increase versus [NADP\(^+\)] is displayed in Fig. 5 and shows saturation behavior. This saturation behavior will only occur if NADP\(^+\) binding occurs in two kinetically distinct steps, perhaps coenzyme binding is followed by a conformational change or formation of the charge-transfer complex, and the reaction can again be described using Scheme 1. Therefore, the data in Fig. 5, main panel, were fitted using Equations 3 and 4 with \(k_f = 89 \pm 2 \text{ s}^{-1}\), \(k_r = 0 \pm 2 \text{ s}^{-1}\) (the forward and reverse rates of this “conformational change”), and \(K_{d\text{NADP}} = 0.8 \pm 0.4 \text{ M}\), when \(K_{d\text{DNA}}\) is fixed to 0.12 and 0.17 \(\mu\text{M}\) (values from Fig. 3B). The kinetic \(K_{d}\) for NADP\(^+\) (0.8 ± 0.4 \(\mu\text{M}\)) is significantly smaller than the spectroscopic \(K_{d}\) determined from Fig. 2A (13 ± 5 \(\mu\text{M}\)). However, if Scheme 1 is correct, the spectroscopic \(K_{d}\) should be equivalent to \(k_f/k_r \times K_{d\text{NADP}}\) as it describes the equilibrium between \([E]\text{N}\) and \([E\text{N}^+]\) not between \([E]N\) and \([E-N]\) (which is the case for \(K_{d\text{NADP}}\) from the fit in Fig. 5). The lower limit to the \(k_f/k_r \times K_{d\text{NADP}}\) equilibrium value, calculated from the values from Fig. 5, is 18 \(\mu\text{M}\), which is in reasonable agreement with the 13 ± 5 \(\mu\text{M}\) value from Fig. 2A.

Thermodynamic Analysis of Electron Transfer in AIF-M2 and AIF-M2 Complexes—The binding of DNA to AIF-M2 gives rise to a change in the circular dichroism spectrum of the protein (19), suggesting some structural perturbation of the protein on binding DNA. This might alter the functional properties of the protein in terms of its flavin redox potential and electron transfer kinetics. To investigate the first possibility, potentiometric analysis of the 6-hydroxy-FAD cofactor was used to probe for any perturbation in the mid-point reduction potential of the cofactor on binding these ligands. Reductive titration of AIF-M2 alone with sodium dithionite under anaerobic conditions gives rise to a bleaching of the FAD absorption centered around 430 nm (Fig. 6, inset). Spectral features were fully reversible on oxidation with potassium ferricyanide. During reduction, a plot of absorbance at 430 nm versus potential is best described by a 2-electron reduction, and a fit to Equation 2 yielded a value for \(E_{1/2}\) of 243 ± 5 mV (Fig. 6). The midpoint potentials for the oxidized/semiquinone (\(E_1\)) and semiquinone/hydroquinone (\(E_2\)) couples are not distinguishable (i.e. there is no inflection of the absorption versus potential curve, and no significant development of spectral signatures that could be associated with flavosemiunione in the optical spectra collected during the redox titration). This may reflect that the \(E_2\) potential is more positive than \(E_1\), and thus that negligible amounts of semiquinone are formed at equilibrium. With the
AIF-M2-DNA complex similar spectral changes were observed during reductive titration, and a value for $E_{1/2}$ of $-242 \pm 5$ mV was calculated by fitting absorption versus potential data using Equation 2. Despite the similar value of $E_{1/2}$ for ligand-free and DNA-bound AIF-M2, there is a distinctive difference in the cooperativity of the transition. There are thus likely to be perturbations in the relative midpoint potentials of $E_1$ and/or $E_2$ couples in the DNA-bound complex, although this perturbation still does not result in any significant population of flavosemiquinone at equilibrium (i.e. $E_2$ likely remains considerably more positive than $E_1$). With the AIF-M2-NADP$^+$ complex the $E_{1/2}$ potential is significantly perturbed to a value of $-196 \pm 5$ mV. Additionally, long wavelength absorption changes were observed in this titration on reduction of the 6-hydroxy-FAD with dithionite, suggesting the stabilization of a reduced flavin-NADP$^+$ charge-transfer species (supplemental Fig. S5).

**Kinetics of Electron Transfer in AIF-M2 and AIF-M2-DNA Complexes**—A more detailed analysis of the electron transfer reactions catalyzed by AIF-M2 and the AIF-M2-DNA complex was undertaken using stopped-flow absorption spectroscopy. Photodiode array analysis of the spectral changes accompanying the rapid mixing of either AIF-M2 or the corresponding DNA complex with NADPH and NADH revealed a decrease in flavin absorption at $\sim430$ nm and the development of charge-transfer characteristics for the reduced enzyme-NADP$^+$ complex at long wavelength ($>600$ nm) (Fig. 7 and supplemental Fig. S6). Global analysis of these data indicated that the spectral changes were best described by a two-step reaction with the major spectral changes occurring in the first step ($A \rightarrow B$), with the second step ($B \rightarrow C$) contributing a relatively minor spectral change (Fig. 7, inset).

Single wavelength analysis of reaction transients measured at 430 nm using a photomultiplier afforded a more accurate analysis of flavin reduction rate constants. With AIF-M2 alone, and consistent with photodiode array detection, reductive transients were biphasic with the major spectral change (flavin reduction) occurring in the faster of the two phases. Observed rate constants for the fast phase were dependent hyperbolically on NAD(P)H concentration (Fig. 8) and were thus analyzed by fitting to a simplified version of Equations 3 and 4 with

$$k_r = 102 \pm 13 \text{ s}^{-1} \quad \text{(NADH)}; \quad k_r = 121 \pm 17 \text{ s}^{-1} \quad \text{(NADPH)}; \quad k_k = 5 \pm 14 \text{ s}^{-1} \quad \text{(NADH)}; \quad k_k = 121 \pm 17 \text{ s}^{-1} \quad \text{(NADPH)}$$

Conditions are as follows. Reactions were performed in 50 mM potassium phosphate buffer, pH 8.0, containing 100 mM potassium chloride at 25 °C.
suggesting that both the initial rate of binding and dissociation are similar for both coenzymes. Finally, the rates of the back reaction (AIF-M2red + NAD(P)H → AIF-M2ox + NAD(P)H) are probably not significant with values of 5 ± 14 and 0 ± 17 s\(^{-1}\) with NADH and NADPH, respectively.

**Re-oxidation Kinetics of AIF-M2**—To complete the analysis of the redox chemistry of AIF-M2, we also investigated the re-oxidation of the dihydroflavin form of the 6-hydroxy-FAD cofactor by molecular oxygen. AIF-M2 was initially reduced by NADPH, and the reoxidation kinetics on mixing with molecular oxygen were found to be second order with respect to oxygen concentration (Fig. 9). Assuming that in air-saturated water the oxygen concentration is \(\sim 0.15 \text{ mm}\), the apparent second-order rate constants for AIF-M2 re-oxidation by molecular oxygen are 0.47 ± 0.02 and 0.47 ± 0.01 mm\(^{-1}\) s\(^{-1}\) in the absence and presence, respectively, of bound DNA. Therefore, DNA binding to AIF-M2 has essentially no effect on the re-oxidation kinetics of the enzyme.

**DISCUSSION**

**Biophysical and Biochemical Properties**—We have demonstrated that AIF-M2 has a high affinity for dsDNA and that this activity is antagonistic with the binding of reducing coenzyme NAD(P)H and associated oxidoreductase activity. A reaction scheme consistent with our observed binding and stopped-flow studies of the AIF-M2 redox chemistry is presented in Fig. 10, in which binding interactions and redox chemistry are shown. This scheme recognizes the mutually exclusive binding of nicotinamide coenzymes and DNA, but it makes no assumption about the mechanism or structural origin of these binding activities (e.g., competitive binding to a common site or binding to nonoverlapping sites). A key aspect of the scheme, however, is the role of dsDNA in regulating the oxidoreductase activity of AIF-M2. Thus, DNA binding activity imparts control on the concentration of ROS that accumulate as products of the AIF-M2 oxidoreductase activity by inhibiting the binding of NAD(P)H and subsequent reduction of 6-hydroxy-FAD.

The cellular NADPH:NADH ratio is \(\sim 4\) (28), and their total concentration has been estimated to be in the order of 100 nm (29). Cellular concentrations of NAD(P)H are thus never likely to approach the apparent \(K_a\) values for the AIF-M2-coenzyme complex (62 ± 18 and 50 ± 13 \(\mu\text{M}\) for NADH and NADPH, respectively). Therefore, in the absence of DNA, the rate of AIF-M2 reduction by low concentrations of NAD(P)H will be approximately second order with rate constants \(~ 2 \text{ \mu M}^{-1} \text{s}^{-1}\) \((k_{\text{obs}} \sim k_{\text{red}}/K_d\text{[NAD(P)H]})\). With a cellular concentration of NAD(P)H of \(~ 100 \text{ nm}\) (29), the rate of AIF-M2 reduction by either coenzyme will then be \(~ 0.2 \text{ s}^{-1}\) and not significantly rate-limited by the re-oxidation kinetics of AIF-M2 by dissolved oxygen \((< \sim 0.1 \text{ s}^{-1})\).

The concentration of DNA in the cytosol is very low in a healthy cell so, despite the ~500-fold difference between the apparent \(K_d\text{[NAD(P)H]}\) and \(K_d\text{[DNA]}\) values, \(K_d\text{[NAD(P)H]} \times [\text{DNA}]\) and reduction of AIF-M2 by NAD(P)H will occur (see above). However, even relatively low concentrations of cytosolic DNA \((>1:\text{500 of cellular [NAD(P)H]}\) will essentially “switch off” the reaction between AIF-M2 and coenzyme as the DNA will outcompete the binding of NAD(P)H to AIF-M2. Consequently ROS production by AIF-M2 will be switched off in the presence of DNA.

The Biological Relevance of Regulating AIFM-2 Activity through Cytosolic DNA Binding—A question that arises is the physiologic consequence of regulating AIF-M2 activity, and the consequent levels of ROS, by DNA binding in the cell. It has been shown that superoxide and hydrogen peroxide are required for growth factor-dependent survival pathways as well as for cell adhesion and immune function (30–32). Knockouts of the aif gene encoding the AIF-M2-related flavoprotein AIF in two human carcinoma cell lines leads to the production of less ROS, loss of tumorigenicity, and increased sensitivity to peroxidase and drug-induced apoptosis (17). These observations have led to the suggestion that AIF knock-out cells are more vulnerable to stress as a consequence of there being lower superoxide and hydrogen peroxide levels in the cell (17). These authors attribute these effects to lessened survival signaling by Ras, NF-κB, or AP-1 (32–34) as a consequence of reduced ROS con-
centrations. This is consistent with the promotion of apoptosis in diverse tumor cell types when ROS levels are reduced (17, 35–37) and the proposed protective role of ROS generated by NAD(P)H oxidases such as AIF and AIF-M2 in healthy cells. As with AIF-M2, the DNA binding activity of AIF also presents an opportunity to regulate the production of ROS through control of AIF oxidoreductase activity.

From our detailed biophysical characterization of the AIF-M2 protein, we propose that AIF-M2, and by inference AIF, regulates the level of ROS species through the DNA binding activities. In healthy cells, which essentially lack cytosolic dsDNA, the natural oxidoreductase activity of AIF (and/or associated complex I activity (17)) and AIF-M2 will generate sufficient levels of ROS to maintain survival signaling. In certain situations cytosolic nucleic acid will be present, for example, during bacterial or viral infection (or alternatively following leakage of mitochondrial DNA during apoptosis). Indeed, onset of apoptosis is seen following viral and bacterial infection of a number of cell types (38–42), although the precise mechanisms of triggering apoptosis are not fully delineated. These mechanisms are likely multifaceted, and we suggest that regulation of ROS levels through DNA binding to AIF-M2 can contribute to this onset of apoptosis in infected cells.

The introduction of double-stranded RNA into the cytosol leads to an activation of type 1 interferon production mediated by the RNA helicases RIG-1 and MDA5 (43, 44). RIG1 in particular is known to be essential for the type 1 interferon response following infection by a number of diverse RNA viruses (45, 46), thus contributing to an antiviral response. Recently, potent type 1 interferon production was also shown in response to cytosolic DNA without detectable activation of NF-κB or mitogen-activated protein kinases, thus demonstrating that the signaling pathways activated by cytosolic DNA are distinct from those activated by cytosolic RNA (20). Under similar conditions of increased cytosolic DNA content following bacterial or (retro)viral infection, we suggest the suppression of AIF-M2 activity through DNA binding will lessen survival pathway signaling as a consequence of reduced ROS, thus potentially contributing to the onset of apoptosis in infected cells. Such a model would require that AIF-M2 has a physiological role in preventing apoptosis. As with AIF, knock out of AIF-M2 protein expression has no effect on both normal and tumor cell viability (23), thus pointing to other sources of ROS to maintain ROS production for survival signaling (17). With AIF knock-outs there is increased sensitivity to peroxide-induced apoptosis, but comparable studies with AIF-M2 knock-outs have not been reported. Also, overexpression of AIF-M2 acts as a potent inducer of apoptosis when there is no DNA in the cytosol (18), but the levels of expressed protein are likely to be much higher than those found in nontransformed cells. This would contribute to relatively high levels of ROS (beyond those required for survival signaling) through the natural redox chemistry of AIF-M2 contributing to intracellular oxidative stress. As with AIF (17), the survival function of AIF-M2 could be dominant over its death inducing activity under normal cellular conditions.

The binding parameters and kinetic data we have obtained for AIF-M2 are consistent with a role in survival signaling at cellular concentrations of AIF-M2, nicotinamide coenzyme, and DNA in healthy and infected cells. Our model establishes a mechanistic link between dsDNA binding, ROS production, and the oxidoreductase activity of AIF-M2 (and by inference AIF). The model provides a mechanistic framework that will facilitate future cell-based studies aimed at clarifying further the cellular functions of AIF-M2 and in relation to viral/bacterial infection and the onset of programmed cell death.

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