The Human Apoptosis-inducing Protein AMID Is an Oxidoreductase with a Modified Flavin Cofactor and DNA Binding Activity*

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AMID (apoptosis-inducing factor-homologous mitochondrial-associated inducer of death; also known as PRG3 (p53-responsive gene 3)) is a human caspase-independent pro-apoptotic protein with some similarity to apoptosis-inducing factor. AMID was purified from a recombinant bacterial host, enabling biochemical analysis of the protein. AMID is a flavoprotein; possesses NAD(P)H oxidase activity; and catalyzes NAD(P)H-dependent reduction of cytochrome c and other electron acceptors, including molecular oxygen. NADPH binds ~10-fold tighter than NADH. AMID binds 6-hydroxy-FAD (a cofactor that accumulates only adventitiously and at low abundance in other flavoprotein enzymes) to form a stoichiometric cofactor-protein complex. AMID has a distinctive electronic spectrum due to the modified flavin. NAD(P)H binding perturbed the spectrum, enabling determination of $K_d$ values for these coenzymes. 6-Hydroxy-FAD could be removed from AMID and the apoprotein reconstituted with FAD. FAD was converted to 6-hydroxy-FAD in reconstituted AMID during aerobic turnover with NADPH. AMID is a DNA-binding protein that lacks apparent DNA sequence specificity. Formation of the protein-DNA complex (i) effected a major protein conformational change and (ii) was prevented in the presence of nicotinamide coenzyme. ApomAMID retains DNA binding activity. Our studies establish a link between coenzyme and DNA binding that likely impacts on the physiological role of AMID in cellular apoptosis.

Apoptosis (or programmed cell death) is an autoregulatory process by which defective cells within tissues of multicellular organisms self-destruct. Apoptosis occurs via either a caspase-dependent or caspase-independent mechanism (1–7). Apoptosis-inducing factor (AIF)§ is a flavin-containing oxidoreductase released from mitochondria during caspase-independent apoptosis (2, 5, 8–11). Caspase-independent apoptosis ensues following the release of AIF from the mitochondrion and after its translocation to the nucleus, where AIF, possibly together with cyclophilin A (12), initiates chromosomal condensation, margination, and degradation.

The human pro-apoptotic protein AMID (apoptosis-inducing factor-homologous mitochondrial-associated inducer of death; also designated PRG3 (p53-responsive gene 3)) was identified recently (13, 14) on the basis of its amino acid sequence similarity (22% identity) to AIF. AMID is also thought to be a caspase-independent effector of apoptosis (14), but, unlike AIF, lacks a mitochondrial localization signal and either is located in the cytoplasm (13, 14) or is associated with the outer mitochondrial membrane (14). The N-terminal domains of both AMID and AIF, which contain putative FAD-binding motifs, can be mutated or deleted without apparent effects on the apoptogenic activity when overexpressed in human cell lines (13–16).

AMID appears to be confined to eukaryotes, with orthologs identified in mammals (Mus musculus; 90% identity), birds (Gallus gallus; 68% identity), amphibians (Xenopus laevis; 61% identity), and fish (Fugu rubripes; 56% identity) and more primitive organisms such as sea squirt (Ciona intestinalis; 37% identity). AMID contains a putative Rossmann fold associated with dinucleotide binding and shares some sequence identity with bacterial oxidoreductases, including Escherichia coli NAD$^+$ reductase and Pseudomonas putida putidaredoxin reductase.

AMID expression is regulated by p53, which binds to p53-responsive elements in the AMID promoter region (13, 17). However, AMID-induced apoptosis is not p53-dependent, as demonstrated by overexpression of AMID in p53-deficient colon cancer cells (14). AMID mRNA levels were recently reported to be down-regulated in tumor tissues compared with matched human normal tissues, suggesting a potential role for AMID in tumor suppression (17). However, an earlier report suggested that AMID mRNA levels are up-regulated in colon cancer cell lines, with levels undetectable in various normal tissue samples (10). During apoptosis, AIF binds to nuclear DNA and is associated with chromatin condensation and DNA fragmentation (18). Mutants of AIF that do not bind DNA following targeted mutagenesis of positively charged surface residues also fail to induce apoptosis (19). Electron microscopy of AMID-expressing 293T cells indicates margination and condensation of chromatin (14) and points to the possibility that AMID might also interact directly or indirectly with DNA.

Herein, we report the biochemical and enzymological properties of purified human AMID. We show that AMID is an NAD(P)H-dependent oxidoreductase and that unusual spectral features of the protein are attributed to the presence of bound 6-hydroxy-FAD, which is associated noncovalently and stoichiometrically with AMID. We demonstrate that AMID is a DNA-binding protein with an apparently sequence-independent mode of interaction, consistent with a proposed role in
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chromatin condensation. We also establish a link between nicotinamide coenzyme binding, protein conformational change, and the ability to bind DNA that we suggest is of physiological significance in the apoptotic function of AMID.

Experimental Procedures

Construction of an AMID Expression Vector—A human cDNA clone containing the AMID open reading frame (IMAGE 3506308; GenBank®/EBI accession number NM_032797) was obtained from Medical Research Council Geneservice (Babraham, UK). The AMID coding sequence was PCR-amplified using Pfu Turbo DNA polymerase (Stratagene) from the cDNA using forward primer 5′-cat atg ggg gcc tgt ggt gaa tcg ecg cca gca gtc cgt ccc aac, incorporating NdeI and BamHI restriction sites (underlined), respectively. The PCR (50 µl) contained 50 ng of cDNA clone, 20 pmol of each primer, 5 µl of 10× Pfu Turbo PCR buffer, 0.2 mM dNTPs, and 3 units of Pfu Turbo DNA polymerase. Cycling parameters were 30 cycles at 95 °C for 30 s, 63 °C for 1 min, and 72 °C for 2 min. The resultant fragment was then cloned into NdeI- and BamHI-digested pET15b (Novagen) to yield an N-terminally hexahistidine-tagged expression clone of AMID. The N-terminal sequence of the His-tagged protein was determined under similar conditions with AMID at a constant final concentration of oxidized pyridine nucleotide coenzymes (NAD(P)H concentration data were analyzed using Origin software (MicroCal).

Titrations were done in the range of 0–1 mM for both NAD(P)H at 0–500 µM.

Circular Dichroism—To examine DNA binding by AMID by gel retention assay, 1 µg of a 100-bp DNA ladder ranging from 100 to 1500 bp (New England Biolabs Inc.) was added to 50 µM of purified AMID and YeG7 (a flavoprotein from Bacillus subtilis) and incubated at room temperature for 30 min. Protein/DNA mixtures were loaded onto 3% agarose gels containing 0.5 mg of purified AMID or AMID (with the tag removed from AMID by digestion overnight with 5 units of thrombin/mg AMID at 16 °C prior to incubation with DNA) were used in the assay. Protein/DNA mixtures were loaded onto 3% agarose gels containing 0.5 µg/ml ethidium bromide and electrophoresed as described (19). To examine the influence of binding of pyridine nucleotides upon binding of DNA by the gel retention assay, AMID (13 µg) was preincubated with NADP⁺ (0–17 mM) and DNA (1.5 µg) for 30 min at 25 °C. The amount of AMID used was 10% in excess of the minimum required to retain all DNA in the well in the NADP⁺-free sample. Thereafter, samples were electrophoresed and analyzed as described above. To examine the ability of 6-hydroxy-FAD-free AMID to bind DNA, apo-AMID was generated on Ni-NTA resin by adding 1 µg of a 100-bp DNA ladder ranging from 100 to 1500 bp (New England Biolabs Inc.) to 50 µM of purified AMID and YeG7 (a flavoprotein from Bacillus subtilis) and incubated at room temperature for 30 min. Protein/DNA mixtures were loaded onto 3% agarose gels containing 0.5 µg/ml ethidium bromide and electrophoresed as described (19). To examine the influence of binding of pyridine nucleotides upon binding of DNA by the gel retention assay, AMID (13 µg) was preincubated with NADP⁺ (0–17 mM) and DNA (1.5 µg) for 30 min at 25 °C. The amount of AMID used was 10% in excess of the minimum required to retain all DNA in the well in the NADP⁺-free sample. Thereafter, samples were electrophoresed and analyzed as described above. To examine the ability of 6-hydroxy-FAD-free AMID to bind DNA, apo-AMID was generated on Ni-NTA resin as described above. Apo-AMID was eluted from the column using imidazole as described above. Protein-containing fractions were identified spectrophotometrically (by the absorption maximum at 280 nm), and fractions containing the major proportion of apo-AMID were pooled, concentrated by ultrafiltration, and gel-filtered to remove imidazole as described above. Apoprotein concentration was estimated using an extinction coefficient of ε280 = 18,610 M⁻¹ cm⁻¹ (using us.expasy.org/tools/protParam.html). The gel retardation assay (as described above for flavin-bound AMID) was repeated with apo-AMID.

Assessment of Protein Conformation and AMID Binding to DNA by Circular Dichroism—The interaction of AMID with DNA was analyzed by measuring the far-UV CD spectrum (190–260 nm) of AMID (5 µM) in the presence and absence of DNA (2 mg of a DNA ladder). The CD spectra of AMID and DNA were measured individually, and then a spectrum of an equal volume mixture of AMID and DNA was measured.

Isolation of the AMID Cofactor—The flavin cofactor was extracted from AMID by protein precipitation with 5% trichloroacetic acid, followed by centrifugation at 13,000 rpm in a microcentrifuge at 4 °C for 10 min in the dark. The cofactor was then lyophilized and reconstituted in 82% (NH₄)₂CO₃ and 18% methanol (pH 8.5) prior to reverse-phase high performance liquid chromatography (HPLC) purification using a Luma C₁₂ column (1 mm × 15 cm) attached to an Agilent Series 1100 HPLC purifier equipped with a UV absorbance detector (at 427 nm). The sample was eluted isocratically using the resuspension buffer. The flow rate was 0.25 ml/min. The instrument was set to collect fractions with absorbance peaks at 260 and 427 nm. The concentration of the protein-free cofactor (subsequently identified as 6-hydroxy-FAD) was determined using an extinction coefficient of ε₄₂₇ = 22.6 mM⁻¹ cm⁻¹ at 427 nm at pH 9.20.

Spectroscopy and Analysis of Purified 6-Hydroxy-FAD—The molecular mass of the AMID cofactor was determined using a Q-Tof Ultima global mass spectrometer (Waters Ltd). MALDI mode was used with positive ions analyzed. The matrix used was a mixture of anthranilic acid, nicotinic acid, and ammonium citrate (27.9 mg of anthranilic acid, 12.3 mg of nicotinic acid, 500 µl of acetonitrile, 300 µl of 100 mM ammonium citrate, and 300 µl of distilled water). Ions were selected in the quadrupole; the collision gas used was argon; collision energies were varied in the range of 15–30 eV; and product ions were analyzed in the time-of-flight analyzer in V-mode. The cofactor sample was dissolved in methanol and “ZipTipped” (using a Millipore C₁₂ ZipTip) prior to mixing in a 1:1 (v/v) ratio with the matrix.

DNA Gel Retention Assay—To examine DNA binding by AMID by gel retention assay, 1 µg of a 100-bp DNA ladder ranging from 100 to 1500 bp (New England Biolabs Inc.) was added to 50 µg of purified AMID or YeG7 (a flavoprotein from Bacillus subtilis) and incubated at room temperature for 30 min. Protein/DNA mixtures were loaded onto 3% agarose gels containing 0.5 mg of purified AMID or AMID (with the tag removed from AMID by digestion overnight with 5 units of thrombin/mg AMID at 16 °C prior to incubation with DNA) were used in the assay. Protein/DNA mixtures were loaded onto 3% agarose gels containing 0.5 µg/ml ethidium bromide and electrophoresed as described (19). To examine the influence of binding of pyridine nucleotides upon binding of DNA by the gel retention assay, AMID (13 µg) was preincubated with NADP⁺ (0–17 mM) and DNA (1.5 µg) for 30 min at 25 °C. The amount of AMID used was 10% in excess of the minimum required to retain all DNA in the well in the NADP⁺-free sample. Thereafter, samples were electrophoresed and analyzed as described above. To examine the ability of 6-hydroxy-FAD-free AMID to bind DNA, apo-AMID was generated on Ni-NTA resin as described above. Apo-AMID was eluted from the column using imidazole as described above. Protein-containing fractions were identified spectrophotometrically (by the absorption maximum at 280 nm), and fractions containing the major proportion of apo-AMID were pooled, concentrated by ultrafiltration, and gel-filtered to remove imidazole as described above. Apoprotein concentration was estimated using an extinction coefficient of ε₄₂₇ = 18,610 M⁻¹ cm⁻¹ (using us.expasy.org/tools/protParam.html). The gel retardation assay (as described above for flavin-bound AMID) was repeated with apo-AMID.

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Differences in CD were computed by comparison of the spectra derived by arithmetical (0.5 times the sum of the individual spectra) and physical (AMID/DNA mixture) addition of the components. Spectra were collected on a Jasco J-715 spectropolarimeter at 25 °C using a 0.02-cm path length quartz cell.

RESULTS AND DISCUSSION

Expression, Purification, and Spectral Analysis of AMID—
AMID was expressed in E. coli HMS174 (DE3) predominantly as a soluble protein to levels of 0.5–2 mg/g of cell paste. The His-tagged protein was purified to homogeneity by a single column chromatography step on Ni-NTA resin as determined by SDS-PAGE analysis (Fig. 1, inset). The protein had electrophoretic mobility consistent with its predicted mass of 42.7 kDa (including the His tag and intervening amino acids). Preliminary studies of the protein revealed a tendency of AMID to aggregate and precipitate in the presence of organic buffers (e.g. Tris-HCl and MOPS) or at low ionic strength. AMID was found to remain stable in solution at slightly alkaline pH (pH 8.0) in phosphate buffer. For assays and spectroscopic analysis, AMID was maintained in 50 mM potassium phosphate containing 300 mM potassium chloride.

As a predicted flavin-binding protein, AMID was expected to have a yellow/orange color. However, AMID was instead found to have a green color, likely indicating a modification of the flavin.

FIG. 1. Purification and UV-visible spectrum of AMID. The UV-visible spectrum of the oxidized form of AMID (50 μM; solid line) is shown overlaid with the spectrum of a “typical” oxidized FAD-containing protein (the B. subtilis oxidoreductase YcgT; 50 μM; dashed line). The absorption maxima of AMID are at 430 and 600 nm. The broad longer wavelength band is a charge transfer transition. The spectrum of YcgT shows two absorption maxima at 378 and 459 nm. Inset, SDS-polyacrylamide gel showing purified AMID protein (lane 1) alongside protein markers (lane 2). Markers visible from the top to bottom have masses of 212, 158, 116, 97, 66, 56, 43, 36, 27, and 20 kDa. AMID has electrophoretic mobility similar to that of the 43-kDa marker.

FIG. 2. Mass spectrometry of the AMID cofactor. A, shown are the MALDI-TOF mass spectra of the AMID cofactor (upper spectrum) and of FAD (lower spectrum). A predominant ion at m/z 802 was present for the AMID cofactor, consistent with the hydroxylation of FAD (786 + 16 = 802). B, precursor ions from the original mass spectra were fragmented and analyzed in a second stage of mass spectrometry. The upper spectrum is for FAD, and the lower spectrum is for the AMID cofactor. For the AMID cofactor, the ions at m/z 259 (243 + 16) and 456 (440 + 16) represent the hydroxylation of the isoalloxazine ring and riboflavin monophosphate moieties of FAD, respectively.

FIG. 3. Interaction of AMID and FAD-bound AMID with reduced pyridine nucleotides. A, UV-visible spectra for oxidized AMID (20 μM; thin solid line) and the NADPH-reduced form (dashed line). Spectra for the NADPH- and NADH-reduced forms of AMID are virtually indistinguishable and have a broad long wavelength absorption band that is maximal between ~670 and 690 nm. Also shown are the oxidized (thick solid line) and NADPH-reduced (dotted line) forms of FAD-reconstituted AMID (20 μM). The spectra for the NADPH- and NADH-reduced forms are, again, virtually indistinguishable, having a broad long wavelength band with an absorption maximum between ~700 and 710 nm. NADPH was added to a final concentration of 100 μM. B, spectra for FAD-bound AMID (16.7 μM; dashed line) and for the same sample (solid line) following reduction by NADPH and reoxidation of the flavin (see “Experimental Procedures”). The re-formation of a considerable amount of 6-hydroxy-FAD is evident from the development of long wavelength absorption and from the peak at ~430 nm. The inset shows the structure of the mesomeric form of 6-hydroxy-FAD as bound by native AMID.

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bound cofactor. The cofactor was released readily from the enzyme by acid precipitation or by heat treatment at 80 °C, indicating noncovalent association with AMID. The UV-visible spectrum of the purified oxidized protein revealed an atypical spectral signature with respect to regular flavoproteins. The absorption spectrum of oxidized AMID (Fig. 1, solid line) has a sharp peak at ~430 nm and a broad long wavelength feature with a peak at ~600 nm. Absorption shoulders are present at ~412 and ~475 nm. Extended incubation in air produced no noticeable change in the electronic absorption spectrum, indicating that the long wavelength transition did not derive from a neutral semiquinone flavin form. The spectrum is completely different from that of a typical flavoprotein. Fig. 1 shows the spectrum of AMID overlaid with that of the FAD-binding oxidoreductase YcgT from B. subtilis, which contains a FAD cofactor with absorbance maxima at 378 and 459 nm (Fig. 1, dashed line). A review of the literature showed that the oxidized spectrum of AMID resembles that of 6-hydroxy-FAD or 6-mercaptoprotopor (FAD, 20, 22, 23). Only the former occurs naturally in proteins. The complete lack of spectral features typical of a non-modified flavin indicates that AMID binds the modified cofactor stoichiometrically. Subsequent HPLC purification of the modified cofactor confirmed that only one cofactor species is associated with AMID.

Characterization of the Modified FAD Cofactor in AMID—
The cofactor was released from AMID by heat inactivation of the protein, which led to precipitation of AMID with the cofactor remaining in solution. The cofactor was purified by reverse-phase HPLC as described under “Experimental Procedures.” Spectral analysis of the unbound cofactor showed that the peak at 430 nm observed in the protein-bound form was blue-shifted to 427 nm. In acidic solutions (e.g. following precipitation of AMID with trichloroacetic acid), the color of the cofactor changed from green to yellow, and the broad absorption band at 600 nm was lost. This is consistent with protonation of the anionic form of either 6-hydroxy-FAD or 6-mercaptopor, resulting in the yellow neutral form (20, 24). In AMID, the cofactor was present in the mesomeric, anionic green form across the pH range of 5.5–8.75 (data not shown).

The purified cofactor was subjected to tandem mass spectrometry to determine whether the cofactor was the anionic form of 6-hydroxy-FAD or 6-mercaptopor. A sample containing purified FAD was also analyzed as a reference. Following MALDI-TOF mass spectrometry, an ion at m/z 802 was obtained for the modified cofactor relative to one at m/z 786 for FAD (Fig. 2A), indicating the presence of an additional oxygen atom in the AMID cofactor and consistent with its being anionic 6-hydroxy-FAD. To confirm that the oxygenation occurred on the isoalloxazine ring, the precursor ions were selected, fragmented, and analyzed in a second stage of mass spectrometry. The cofactor mass spectrum shown in Fig. 2B indicates the presence of two ions at m/z 259 and 456, which differ by 16 units from the ions at m/z 243 (isoalloxazine ring) and 440 (riboflavin monophosphate) obtained for FAD. This provides further evidence that the isoalloxazine ring is the site of substitution and that the cofactor bound by AMID is 6-hydroxy-FAD. On the basis of the previously determined extinction coefficient of ε247 = 22,600 M⁻¹ cm⁻¹ for free 6-hydroxy-FAD at pH 9.0 (19), we calculated an extinction coefficient of ε430 = 25,500 M⁻¹ cm⁻¹ for AMID-bound 6-hydroxy-FAD under our experimental conditions at pH 8.0.

A number of enzymes have now been purified in which the FAD cofactor exists partially in a form that is hydroxylated at C-6 of the isoalloxazine ring (20, 22, 25–28). In many cases, this yields a catalytically inactive enzyme or one with reduced activity compared with the FAD-containing protein and represents only a small percentage of the total flavin content. However, to our knowledge, AMID is the first protein characterized in which 6-hydroxy-FAD is bound stoichiometrically. Through heat treatment, we were able to remove completely 6-hydroxy-FAD bound to AMID and subsequently to reconstitute the immobilized apoprotein with natural FAD. The spectra of the FAD- and 6-hydroxy-FAD-containing forms are clearly distinct, although both were reduced to their hydroquinone forms upon addition of NADH/NADPH, without obvious formation of any semiquinone species (Fig. 3A). However, for FAD-reconstituted
AMID, reduction with both NADH and NADPH leads to the development of long wavelength absorption possibly consistent with development of a charge transfer species between the hydroquinone flavin and the oxidized nicotinamide cofactor (29). For AMID itself, reduction by NAD(P)H also leads to development of a long wavelength band distinct from that seen in the oxidized form. Again, we consider that this reflects the formation of a charge transfer species that is distinct from the mesomeric, anionic form of 6-hydroxy-FAD, the latter providing the long wavelength absorption feature seen for oxidized AMID (Fig. 3A, inset).

With FAD-reconstituted AMID, we observed a partial conversion from FAD to 6-hydroxy-FAD following reduction by NADPH and subsequent reoxidation of AMID (Fig. 3B). Progressive aggregation and precipitation of reconstituted AMID protein were observed over time in the presence of NADPH, which prevented the continued reduction and reoxidation cycles that may have led to more complete formation of 6-hydroxy-FAD. In parallel reduction/oxidation studies of FAD-reconstituted AMID using NADH as reductant, no development of a spectral signature indicative of the formation of 6-hydroxy-FAD was observed (but progressive AMID aggregation did take place). Although we cannot rule out absolutely that formation of some 6-hydroxy-FAD may occur as a result of extensive redox cycling with NADH, the reaction is clearly much less efficient than that with NADPH. Pyridine nucleotide-binding enzymes that discriminate in favor of NADPH over NADH usually have a discrete binding site for the additional 2′-phosphate group on NAD(P)H (e.g. Ref. 29). Thus, a distinctive binding mode for NADPH that enhances its ability (over NADH) to induce redox-dependent hydroxylation of FAD cannot be ruled out at this stage. These findings are consistent with the data presented below showing superior binding and kinetic properties with NADPH.

AMID Is an NAD(P)H-dependent Oxidoreductase—In view of the data above indicating NADPH-dependent formation of 6-hydroxy-FAD in AMID, we undertook steady-state kinetic studies to examine the capacity of AMID to catalyze oxidation of NAD(P)H and electron transfer to various electron acceptors (oxygen, ferricenium hexafluorophosphate, potassium ferricyanide, and cytochrome c). The data are shown in Table I. These data demonstrate that AMID catalyzed dehydrogenation of both reduced pyridine nucleotides and was able to pass electrons both to molecular oxygen and to other acceptor molecules. AMID displayed a clear preference for NADPH over NADH (from ∼7 to ∼12.5-fold, depending on the nature of the final electron acceptor used) (Table I), with an apparent $K_m$ of ∼3 μM for NADPH when using oxygen, ferricenium, and ferricyanide as electron acceptors. A catalytic rate ($k_{cat}$) of ∼5000 min$^{-1}$ was observed with ferricyanide as the electron acceptor and either NADH or NADPH as the donor, indicating that the limiting reduction rate ($k_{red}$) of the AMID flavin by either coenzyme is >40 s$^{-1}$. Reduction of molecular oxygen was relatively slow (<2 min$^{-1}$), apparently ruling out any role for AMID as a generator of oxygen radicals. Interestingly, AMID bound cytochrome c quite tightly ($K_d$ = 7.4 μM) and reduced this apoptosis-related protein efficiently.

Interaction of NAD(P)$^+$ with AMID—Preliminary experiments indicated that perturbation of the optical spectrum of AMID occurred upon addition of aliquots of the oxidized pyridine nucleotide coenzymes NAD$^+$ and NADP$^+$. This finding was exploited to determine the apparent $K_d$ values for NAD(P)$^+$ binding to oxidized AMID (Fig. 4). Spectral titrations

**Fig. 5.** AMID binding to DNA. A, gel retention assays were performed on 3% agarose gels with His-tagged AMID (50 μg) following incubation with 1 μg of the 100-bp DNA marker (lane 1) or with non-His-tagged AMID (50 μg, lane 2). Failure of the DNA marker to resolve is indicative of DNA binding to AMID and retention in the gel well. Negative controls comprising the DNA marker incubated with the NADP(H)-binding flavoprotein YcgT from B. subtilis (50 μg, lane 3) and the DNA marker alone (1 μg, lane 4) did not result in retardation of DNA marker migration. B, the conditions were as described for A, but His-tagged AMID (13 μg) was preincubated with NADP$^+$ (0–17 mM) and DNA (1.5 μg). Lanes 1–6, NADP$^+$ at 17, 13, 10, 7, 3, and 2 mM, respectively; lane 7, no NADP$^+$; lane 8, DNA marker alone. Release of DNA occurred progressively as the NADP$^+$ concentration was increased. C, far-UV CD spectra were recorded for AMID in the presence and absence of DNA to determine whether a conformational change occurred following DNA binding. The combined far-UV CD spectra consisting of the individual spectra obtained for AMID alone and DNA alone (dashed line) and the actual spectrum recorded for DNA-bound AMID (solid line) are depicted. The differences in the shapes and intensities of the two curves indicate that a conformational change had occurred following binding of AMID to DNA, deg, degrees.
were performed as described under “Experimental Procedures.” $K_d$ values of $17.5 \pm 1.1 \mu M$ (NADP$^+$) and $249 \pm 9 \mu M$ (NAD$^+$) were determined. These data are consistent with those from steady-state analyses (see above), indicating that NAD$^+$ bound $\sim$14-fold weaker to oxidized AMID than did NADP$^+$. Moreover, from comparisons of these $K_d$ data with the $K_m$ data from kinetic studies (using parameters from ferricyanide reduction studies as an example), both NAD$^+$ ($\sim$6.2-fold) and NADP$^+$ ($\sim$5.5-fold) bound substantially more weakly to AMID than did NADH and NADPH, respectively. Thus, AMID binds reduced pyridine nucleotide coenzymes significantly tighter than their oxidized forms.

AMID Binds to DNA—AMID was incubated with a 100-bp DNA ladder and then subjected to agarose gel electrophoresis. Retardation of DNA migration through the gel because of the formation of an AMID-DNA complex was evident (Fig. 5A, lane 1). To exclude the possibility that AMID might bind to DNA via the positively charged N-terminal His tag, we also performed DNA binding assays with thrombin-treated AMID (which lacks the N-terminal His tag). AMID treated in this way retained its DNA binding capacity (Fig. 5A, lane 2). As expected, neither incubation of a DNA marker with the B. subtilis flavoprotein YcgT and nor incubation of the DNA marker alone resulted in retardation of DNA migration (Fig. 5A, lanes 3 and 4, respectively). The effect of pyridine nucleotide ligand association on DNA binding was also investigated by gel retardation analysis using AMID preincubiated with NAD(P)(H) at various concentrations. AMID was used at a concentration 10% above that required for complete retardation of the DNA in the pyridine nucleotide-free form. DNA release from AMID into the gel was observed at high concentrations of all of the pyridine nucleotides. Fig. 5B shows the inability of AMID to bind DNA when incubated with a variety of concentrations of NADP$^+$. That both oxidized and reduced pyridine nucleotides prevented association with DNA indicates that the ligand-inhibited ability to bind DNA is not related to the flavin redox state. The relatively high concentrations of ligand required (millimolar concentrations) to inhibit DNA binding may indicate overlapping DNA- and NAD(P)(H)-binding sites on the enzyme.

To establish whether association of flavin is important for DNA binding, gel retention assays were repeated using apo-AMID (prepared as described under “Experimental Procedures”). As expected, apo-AMID retained full DNA binding activity. Thus, removal of 6-hydroxy-FAD does not impact significantly on the DNA binding properties of AMID.

Addition of DNA did not perturb the visible spectrum of AMID significantly, consistent with there being distinct DNA- and FAD-binding regions/domains in AMID and with the ability of apo-AMID to bind DNA. To examine further the interactions of AMID with DNA, we used CD spectroscopy to probe for conformational changes. Fig. 5C shows the effect of DNA binding on the secondary structural features of AMID. The spectra are those for the algebraic addition of the spectra for DNA and AMID in isolation and for the physical mixture of AMID and DNA at the same effective concentration. A structural change was induced in AMID as a consequence of DNA binding to the protein.

The gel retention assays and far-UV CD spectra indicate that, like AIF, AMID binds DNA. As with AIF, we have also found no evidence for nuclease activity in vitro (data not shown). Recently, it was shown that AIF interacts with cyclophilin A both by co-immunoprecipitation and pull-down assays and by co-localization in tissue culture cells undergoing apoptosis (12). DNA degradation was found to be dependent upon the presence of both AIF and cyclophilin A in vitro and in vivo, indicating a requirement for complex formation. It will clearly be important to establish whether AMID binds cyclophilin A or other proteins to establish a complex that can catalyze hydrolysis of DNA.

Conclusion—AMID is a flavoprotein with NAD(P)/H-dependent oxidoreductase activity. The protein is assembled stoichiometrically and noncovalently with 6-hydroxy-FAD. NADPH is the preferred coenzyme, and both NADPH and NADP$^+$ bind AMID considerably tighter than do NADH and NAD$^+$. FAD can replace 6-hydroxy-FAD, but aerobic turnover with NADPH leads to formation of 6-hydroxy-FAD. We infer that 6-hydroxy-FAD is the natural cofactor of AMID. AMID binds DNA nonspecifically, and DNA binding leads to a protein conformational change. Flavin-depleted AMID retains DNA binding activity, confirming that the binding sites for DNA and flavin are distinct. However, DNA binding is inhibited by pyridine nucleotide coenzyme association, thus establishing a link between the oxidoreductase and DNA-binding domains that likely impacts on the physiological role of AMID in cellular apoptosis.
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