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Flavin-binding of azoreductase: Direct evidences for dual-binding property of apo-azoreductase with FMN and FAD

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ABSTRACT

The flavin selectivity of the flavoenzymes is considered to be very strict in terms of the functional expression of the enzyme. However, we found that an FMN-dependent azoreductase from *Bacillus* sp. B29 exhibited up to 60% of the activity of native AzrA harboring FMN upon the addition of a FAD cofactor. The FAD binding to the apo-form of AzrA was identified by spectrophotometric analysis, and the bound FAD was stably retained in the enzyme molecule without degradation to FMN. On the other hand, no effect of riboflavin on the activity of AzrA was detected and there was no obvious quenching of riboflavin detected with the addition of apoAzrA. By a docking simulation of FAD into the structure of a homolog of AzrA (AzoR from *Escherichia coli*), we created a FAD-binding model. Taking all of these results together, it is proposed that the isoalloxazine ring of FAD localizes at the same site and plays the same role as that of FMN in AzrA.

1. Introduction

Flavoenzymes are involved in a variety of important biological reactions and comprise wide spread class of enzymes that generally catalyze redox reactions. All flavoenzymes include a covalently or noncovalently bound flavin as a functional cofactor, typically containing either flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD), which have an isoalloxazine ring system which enables them to catalyze both one- and two-electron transfer reactions [1]. Generally, flavoproteins bind to FMN but do not interact with FAD and vice versa. These features may have facilitated the specific binding of flavin species to the apoflavoproteins through the evolution of several conserved protein binding motifs. Therefore, the flavin selectivity of the proteins seems to be very strict in terms of functional expression.

The azoreductases also comprise a class of the flavoenzymes which catalyzes the reductive cleavage of the azo linkage in the azo dyes to produce corresponding aromatic amines [2]. These enzymes play a key role in azo dye-contaminated waste water treatment for bioremediation [2,3]. Flavin-containing azoreductases can be classified into at least two types based on the coenzyme requirement, i.e., either NADH or NADPH as the electron donor [4].

Recently, we isolated and characterized three novel azoreductase homologues from the bacterium *Bacillus* sp. B29, i.e., terminated AzrA, AzrB and AzrC [5,6]. These azoreductases belong to the NADH-requiring azoreductases which contain two noncovalently bound FMNs in the homodimeric protein and display similar catalytic properties as well as the same class of other bacterial azoreductases, such as AzoR from *Escherichia coli* [7], AzoA from *Enterococcus faecalis* [8], azoreductase from *Bacillus subtilis* [9] and AzrG from *Geobacillus stearothermophilus* [10]. They have been shown to reduce azo compounds via a "Ping Pong Bi Bi" mechanism.

During the course of analyzing the flavin specificity of azoreductase, we unexpectedly found that apoAzrA exhibited enzyme activity by reconstituting with FAD, as in the case obtained for FMN. Similar observations were reported for the bacterial azoreductases, that is an enhancement by the addition of external FAD [11,12]. Although these results suggest the possibility of binding non-original flavin species, direct evidence has not been provided.

In this study, we investigated the binding feature of both of the FMN and FAD flavins to AzrA in detail by physicochemical analyses. Intact apoAzrA was successfully prepared from a recombinant *E. coli* strain and used to reconstitute with the flavins. This is a first report that AzrA possesses a binding capacity for both FMN and FAD.

Abbreviations: AzrA, *Bacillus* sp. B29 azoreductase; K_d, dissociation constant; apoAzrA, apo-form of AzrA; holoAzrA, holo-form of AzrA; FMN-AzrA, reconstituted AzrA with FMN; FAD-AzrA, reconstituted AzrA with FAD.

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2. Materials and methods

2.1. Purification and reconstitution of apoAzrA

The detailed purification procedure from the recombinant *E. coli* expressing AzrA was described previously [5]. ApoAzrA was separated from holoAzrA by a hydrophobic interaction chromatography using Toyopearl Butyl-650 column (Tosoh, Japan). The active fractions of DEAE-cellulose column chromatography were applied to a Toyopearl Butyl 650 column (24 mm × 220 mm). The protein was eluted using a linear ammonium sulfate gradient (20–0% saturation) in the 25 mM Tris–HCl buffer (pH 7.4) at a flow rate of 1 ml/min. Each holoAzrA and apoAzrA fractions were separately combined and were further purified by gel filtration on a Sephacryl S-200HR column. For the reconstitution experiment, flavin (riboflavin, FMN, and FAD) solutions were added to the purified apoAzrA at a molar ratio of 100:1 and then stood in 25 mM Tris–HCl buffer (pH 7.4) at 4 °C for 30 min. The excess amounts of flavins from FMN–AzrA and FAD–AzrA were removed using a desalting column (PD-10, GE healthcare) in the same buffer.

2.2. Chemicals and assay methods

Riboflavin (≥98%), FMN (sodium salt, ≥95%), and FAD (disodium salt, ≥94%) were purchased from Sigma (St. Louis, USA). All the other chemicals were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Azoreductase assay was based on the measurement of the reduction rate of methyl red as a substrate at 430 nm as described previously [5]. When apoAzrA was assayed, riboflavin, FMN, or FAD was added into the reaction mixture at the concentration of 25 μM. Purity of FMN and FAD was examined by HPLC analysis. The enzyme purity was examined by SDS-PAGE analysis, and the protein concentration was determined using protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, USA) with bovine serum albumin as a standard. The protein-bound FMN and FAD were calculated using the extinction coefficients of 12,500 M^{-1} cm^{-1} at 445 nm for FMN and 11,300 M^{-1} cm^{-1} at 448 nm for FAD [13].

2.3. Determination of dissociation constants for the complex of apoAzrA with flavins

The K_d value for the binding of riboflavin, FMN, and FAD with apoAzrA was determined by fluorescence titration method as described previously [14]. Microliter quantities of apoAzrA were added to 3 ml aliquots of a 0.1 μM flavin in 25 mM Tris–HCl buffer (pH 7.4) at 25 °C. Quenching of the flavin fluorescence occurred within several minutes. The proportion of bound flavin was determined at equilibrium by measuring the extent of quenching.

2.4. Flavin identification

Enzyme bound flavins were released by addition of ice-cold 5% trichloroacetic acid (TCA) with 0.3 mM EDTA. After 10 min, the precipitated protein was removed by centrifugation at 12,000 × *g* and at 4 °C for 10 min. The resulting supernatant containing flavin cofactor was applied onto a reversed phase HPLC column (TSKgel ODS 100Z, Tosoh, Japan). The solvent system used was 10% acetonitrile in 0.1% trofluoroacetic acid and the flow rate was 1 ml/min. Flavin was monitored at 450 nm.

2.5. Docking simulation

For help to investigate the binding mechanism between FAD and AzrA, 3D-structure prediction of AzrA was firstly performed by using a web service Phyre2 [15]. The result showed that a 3D model of FMN–AzoR complex (PDB code: 2Z9C) could be one of high structural similarity to AzrA. The docking simulation of FAD was therefore carried out by using the structure of AzoR. For the docking process, the data preparation for FAD and AzoR model was performed by using *Ligprep and Protein Prepare Wizard* programs in *Mastro* 8.0 software [16]. All the water molecules and FMN molecules in 2Z9C were removed. Hydrogen atoms were added and H-bonds were meanwhile optimized. The geometric center of FMN in FMN–AzoR complex (2Z9C) was chosen as the center of enclosing box and the sizes of which were set as 20 Å for *X*, *Y* and *Z*. The docking calculation was run in the SP (standard precision) mode with default values for all parameters by using *Glide* [17]. The docking score of −9.0 was used as a threshold to choose the top 10 results.

Table 1
Specific activity of purified holoAzrA and apoAzrA with flavin analogs.

Flavins	Specific activity (units/mg protein)	
	holoAzrA	apoAzrA
None	26.2	ND
Riboflavin	26.8	ND
FMN	26.4	26.9
FAD	26.0	15.4

ND, not detected.

3. Results and discussion

3.1. Purification of apoAzrA and holoAzrA

AzrA was expressed in *E. coli* BL21(DE3)pLysS harboring pET3a–AzrA and purified at pH 7.4 using the same procedure as described previously [5]. During the purification of AzrA, two distinct protein peaks were observed using hydrophobic interaction chromatography, as shown Fig. 1A. The holoAzrA was detected from the first elution protein peak, which exhibited a yellow color corresponding to FMN with the azoreductase activity. The protein from the second elution peak did not exhibit any yellow color and the enzyme activity was not detected under standard assay conditions. The azoreductase activity of the second elution protein was restored by adding FMN to the reaction mixture. As in the case with holoAzrA, the purified protein from the second fraction had a subunit molecular mass of approximately 23 kDa, as determined by SDS-PAGE (Fig. 1B), and a 45 kDa by gel permeation chromatography (data not shown), indicating that the protein is a homodimer, the same as holoAzrA. In addition, spectrophotometric analysis revealed that the purified protein from the second fraction did not display any absorption signals corresponding to a flavin cofactor, as shown in Fig. 1C. These results suggested that the second fraction was the apoAzrA protein.

3.2. Enzyme activity of AzrAs reconstituted by combination with flavin analogs

The enzyme activity of apoAzrA was measured under the standard assay conditions by adding each of the flavin analogs (Table 1). ApoAzrA had the azoreductase activity restored in the presence of FMN and FAD, but not riboflavin. The FMN–AzrA had a specific activity of 26.9 units/mg protein, which is a level comparable to that of holoAzrA. The FAD–AzrA was also active, but exhibited decreased specific activity (15.4 units/mg protein) against methyl red as a substrate, implying the FMN specificity of this enzyme. The specific activity of holoAzrA was not changed by the addition of any of the three flavins to the reaction mixture.

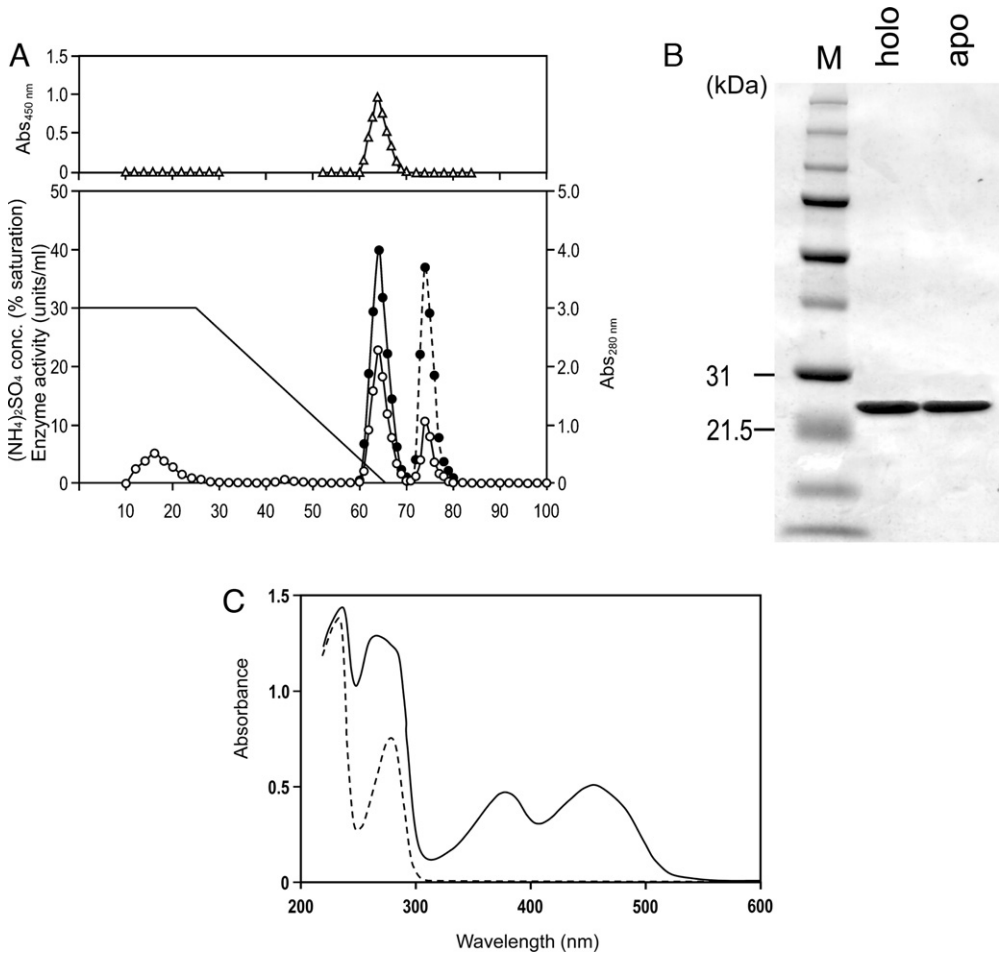

Fig. 1. (A) Separation of holoAzrA and apoAzrA on a Toyopearl Butyl-650 column. Absorptions at 280 nm and at 450 nm are indicated by the open circles and triangles. Enzyme activity is shown by the closed circles. The enzyme activity which was restored by the addition of FMN is indicated by the dashed line. (B) SDS-PAGE of purified AzrA. Lane M, protein markers; lane 1, holoAzrA; lane 2, apoAzrA. Purified enzymes were separated on a 15% SDS-polyacrylamide gel. Each lane was loaded with a sample containing 10 μg of protein. The gel was stained with Coomassie Brilliant Blue R-250. (C) The absorption spectra in the UV–VIS region of purified holoAzrA (solid line) and apoAzrA (dashed line).

3.3. Properties of the reconstituted AzrA with flavin analogs

We examined the association of enzymes with FAD that could function as a cofactor like FMN. First, to analyze the stoichiometric binding of AzrA with the individual flavins, FMN and FAD, the reconstitution of AzrA with FMN and FAD was performed by the incubation the apoAzrA with an excess amount of each of the flavin potential cofactors. After removing the unbound

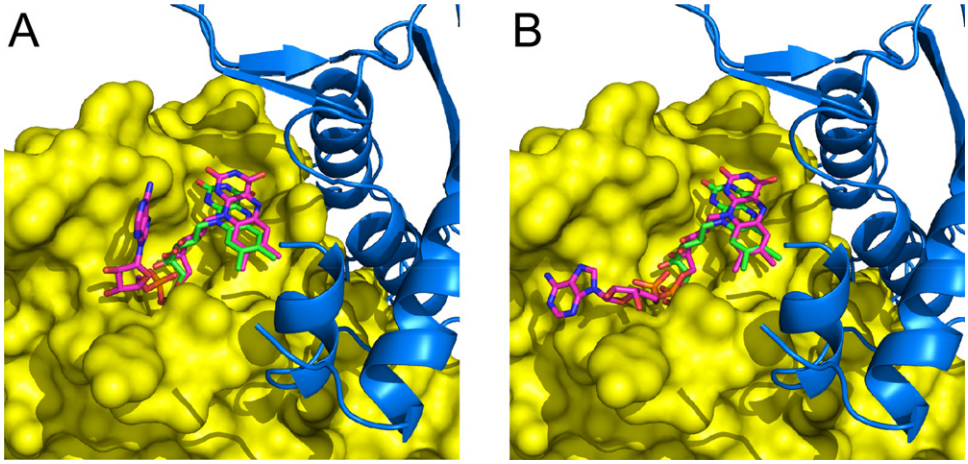

Fig. 4. The docking model of FAD–AzoR. The monomers in the crystallographic structures of AzoR dimer are shown in the surface and blue ribbon model, respectively. The FMN (green) in the crystallographic structure of the AzoR–FMN complex and the docking-model of FAD (C atoms: purple; N atoms: blue; O atoms: red; P atoms: orange) are shown in stick model. The r.m.s.d. values of the FMN moiety in the two simulated models are (A) 1.08 Å and (B) 1.40 Å, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

excess flavins with a desalting column, the protein-bound flavin and the protein amount of the reconstituted AzrAs were quantitated. On the basis of these experimental results, the ratio between the respective flavins and the AzrA protein was calculated to be 2.2 for FMN and 1.9 for FAD. This suggests that FMN–AzrA and FAD–AzrA contained 2 mol of each flavin together with 1 mol of the homodimeric AzrA. The spectrophotometric analyses of the reconstituted AzrAs indicated that there was an apparent change in the absorbance features, as shown in Fig. 2. FMN–AzrA exhibited two distinct absorption maxima at 377 and 456 nm, which were almost identical to those of native holoAzrA [5]. FAD–AzrA also exhibited two absorption maxima at 374 and 457 nm. The spectra of both of the reconstituted AzrAs displayed peak shifts toward a long wave length compared with those of free FMN and FAD. This observation is a typical phenomenon of flavoprotein absorption [13], indicating that each of the flavin cofactors was associated with apoAzrA.

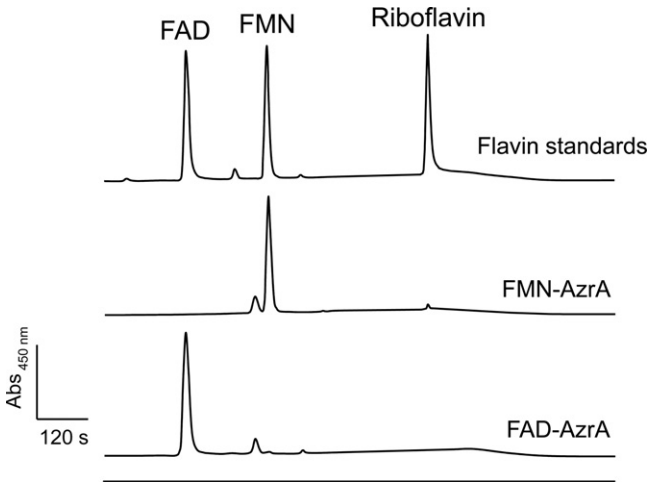

Fig. 3. HPLC identification of flavins from the reconstituted AzrA with FMN (FMN–AzrA) and FAD (FAD–AzrA).

3.4. Identification of bound flavin species in the reconstituted AzrAs

Although FAD was stable under our experimental conditions, it is possible that that the protein-bound FAD would degrade into FMN and adenine monophosphate by an unknown mechanism. To exclude this possibility, we identified the flavin species in the reconstituted AzrA protein. The supernatants containing the released flavin obtained from the reconstituted AzrA after denaturation by ice-cold TCA were analyzed by reversed-phase HPLC (Fig. 3). FMN–AzrA displayed a single peak corresponding to FMN, as in the case of holoAzrA, and FAD–AzrA also displayed a single peak corresponding to FAD. FMN and riboflavin were not detected in FAD–AzrA. These results strongly suggest that FAD was present in each of the reconstituted AzrAs without any flavin degradation, and that FAD was noncovalently bound to the AzrA.

3.5. Dissociation constants of apoAzrA and flavin species

The quenching of flavin fluorescence by apoAzrA was used to determine the dissociation constants for the binding of the flavin species to apoAzrA. As expected, FMN and FAD were able to bind to apoAzrA, but there was no quenching of the flavin fluorescence observed in the case of riboflavin, indicating no binding with the apoAzrA under the experimental condition. The K_d values were determined to be 2.8 nM for FMN and 11.4 nM for FAD, reflecting a good correlation with the enzyme activities for both FMN–AzrA and FAD–AzrA. The FAD cofactor is loosely bound to the enzyme instead of FMN and the flavin binding site is readily accessible to FAD. One explanation might be that the flavin specificity of AzrA is caused by structural features of the flavin analogs. In general, the phosphate groups and the adenylate group of flavin analogs provide the contact for the binding between the protein and the cofactor. Therefore, it is suggested that the phosphate group may play a key role in the recognition and binding between the apoAzrA and the flavins. The importance of the phosphate group in the stable binding between FMN and apoflavodoxin from *Desulfovibrio vulgaris* has been described [18,19]. The phosphate group, with a ribityl side chain of FMN, is anchored by several hydrogen bonds in a phosphate-binding site at the active site of the flavodoxin.

3.6. FAD binding in AzrA

The active redox center of the flavin cofactor is located in the isoalloxazine ring in the flavin analogs. As well as FMN, AzrA-bound FAD has the potential for electron transfer which proceeds by the same mechanism based on the isoalloxazine ring. Although the details of how FAD binds to the apoAzrA and the actions of the isoalloxazine ring and the adenyl phosphate moiety of FAD in AzrA are not presently clear, the isoalloxazine ring might be situated in the intrinsic binding pocket of AzrA, which would provide redox potential to the electron transporting activity that is essential for the azoreductase activity [20].

Two FMN-dependent azoreductases, AzoR of *E. coli* and AzoA of *E. faecalis* are structurally of the same class as AzrA of *Bacillus*

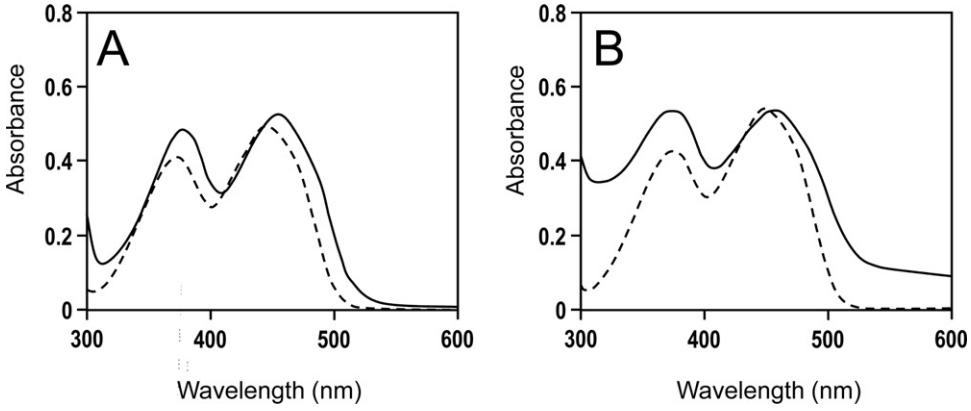

Fig. 2. Absorption spectra of reconstituted AzrA with FMN (A) and with FAD (B). The spectra were recorded for the reconstituted enzymes at the concentration of 1 mg/ml in 25 mM Tris–HCl buffer (pH 7.4) at room temperature. The spectra of the authentic FMN (A) and FAD (B) at a concentration of 40 μM in the same buffer is shown with the dashed lines.

sp. B29, which possesses a homodimeric structure [7,8]. The amino acid sequence of AzrA has 30% and 37% identity with AzoR and AzoA, respectively. The crystal structures of AzoR and AzoA display the same structural topology and have a similar specific binding groove for the ribityl phosphate moiety of FMN [21,22]. Although the amino acid sequence identity is not particularly high, the conformation of the active site in AzrA, including the FMN binding site, would be similar to that in both AzoR and AzoA [21,22].

To investigate the binding of FAD with AzrA, we docked FAD into AzoR to create a binding model, since 3D-structure prediction of AzrA showed that the structure of AzrA is similar to that of AzoR. The results of the docking simulation indicated two primary binding patterns for AzoR–FAD (Fig. 4). In both of the two patterns, the FMN moiety of FAD was located at the same position as that of the crystallographic structure of AzoR–FMN with a root means square difference (r.m.s.d.) of 1.08 and 1.40 Å, respectively, whereas the adenosine moiety of FAD was positioned on the protein surface in a very different orientation. In one pattern, the adenosine moiety of FAD found to be localized close to the protein surface (elongated shape), while in the other one it was extended away from the surface (bent shape). In fact, these two kinds of FAD binding pattern have also been reported in other flavoenzymes [23]. Considering the activity of the AzrA–FAD complex, the docking result suggests that the isoalloxazine ring of FAD plays the same role as that of FMN in the AzoR–FMN complex, and the adenyl phosphate moiety bound on the protein surface may not affect the binding stability of the isoalloxazine ring and AzoR. In order to gain insight into the detailed binding features of the flavin cofactors, it will be necessary to determine the crystal structures of both the AzrA–FMN and AzrA–FAD complexes.

4. Conclusion

This is the first study that has actually demonstrated the dual-binding property of AzrA to two different flavin cofactors, FMN and FAD. Even though AzrA from *Bacillus* sp. B29 is recognized as an intrinsically FMN-dependent enzyme, we have presented direct evidence that AzrA can bind with FAD instead of FMN as a functional cofactor. This finding is based upon the successful preparation of the intact apoAzrA. These results provide a basis for additional future study and will facilitate the identification of molecular determinants of flavin binding. Further spectroscopic and crystallographic studies of AzrA should help clarify the recognition mechanism responsible for the binding of FMN and FAD.

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