A Novel NAD-binding Protein Revealed by the Crystal Structure of 2,3-Diketo-L-gulonate Reductase (YiaK)*

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Escherichia coli YiaK catalyzes the reduction of 2,3-diketo-L-gulonate in the presence of NADH. It belongs to a large family of oxidoreductases that is conserved in archaea, bacteria, and eukaryotes but shows no sequence homology to other proteins. We report here the crystal structures at up to 2.0-Å resolution of YiaK alone and in complex with NAD-tartrate. YiaK has a new polypeptide backbone fold and a novel mode of recognizing the NAD cofactor. In addition, NAD is bound in an unusual conformation, at the interface of a dimer of the enzyme. The crystallographic analysis unexpectedly revealed the binding of tartrate in the active site. Enzyme kinetics studies confirm that tartrate and the related D-malate are inhibitors of YiaK. In contrast to most other enzymes where substrate binding produces a more closed conformation, the binding of NAD-tartrate to YiaK produces a more open active site. The free enzyme conformation is incompatible with NAD binding. His44 is likely the catalytic residue of the enzyme.

In Escherichia coli, yiaK is the first gene in a nine-gene operon (1), yiaKLMNOPQRS (yiaK-S), that is believed to be required for the utilization of rare sugars for growth. Mutational deactivation of the regulator of this operon, yiaJ, located just prior to yiaK but in the opposite orientation, leads to the constitutive expression of all the proteins of the operon, which enables bacteria carrying this mutation to utilize the rare pentose L-lyxose (2). However, not all of the genes of the operon are needed for the metabolism of this sugar. It is believed that L-lyxose is first converted to L-xylulose. Then the YiaP, YiaR, and YiaS proteins, encoded in the second half of the operon, can convert L-xylulose to L-xylulose 5-phosphate, which can in turn enter the pentose phosphate pathway (3). Therefore, it is likely that L-xylulose is only an intermediate from the action of the YiaK-S proteins on the genuine substrate(s) of this operon, which is currently still unknown (4).

The YiaK protein catalyzes the reduction of 2,3-diketo-L-gulonate (DKG) in the presence of NADH, and the product of the reduction is believed to be 3-keto-L-gulonate (see Fig. 1A) (5, 6). DKG can be obtained from the hydrolysis of L-dehydroascorbate. Another operon in E. coli, yif-sga (now known as ula), is involved in the fermentation of L-ascorbate (5). The yiaK-S operon may be involved in the utilization of L-dehydroascorbate.

YiaK belongs to a large family of putative oxidoreductases that have members from bacteria and archaea, as well as eukaryotes (see Fig. 1B). There are four additional homologs of YiaK in E. coli, two of which are shown in Fig. 1B. Many members of this family are annotated as type 2 malate/lactate dehydrogenase in the various sequence data bases, although there is only minimal biochemical evidence supporting this classification. Moreover, members of this family do not share any recognizable sequence homology to other NAD(P)-binding proteins. For example, the YiaK proteins do not contain the GXXGXXG(A/G) dinucleotide binding signature motif (7). As a matter of fact, these proteins do not share any significant sequence homology with any other proteins that have known three-dimensional structures.

To help understand the biochemical functions of these enzymes, we have determined the crystal structures of the free enzyme of YiaK and its complex with NAD-tartrate at up to a 2.0-Å resolution. The structures reveal a new polypeptide backbone fold for the enzyme as well as a novel mode of binding the NAD cofactor, establishing the YiaK enzymes as a new class of NAD(P)-dependent oxidoreductases. In addition, our crystallographic analysis unexpectedly showed the binding of tartrate in the active site. Enzyme kinetics studies confirm that tartrate and the related D-malate are competitive inhibitors of YiaK. Large conformational differences are observed between the free enzyme and the NAD-tartrate complex. In contrast to most other enzymes in which substrate binding produces a more closed conformation, the binding of NAD-tartrate to YiaK produces a more open active site. The conformation of the free enzyme of YiaK is incompatible with NAD binding.

MATERIALS AND METHODS

Expression and Purification of YiaK—The full-length yiaK gene from E. coli, Northeast Structural Genomics Consortium target ER82, was cloned into a PET21d (Novagen) derivative, generating plasmid pER82-21. The resulting recombinant protein contains eight non-native residues (LEHAAAAHI) at the C terminus. The construct was sequence-verified by standard DNA sequence analysis. E. coli BL21 (DE3) pMGK cells, a rare codon-enhanced strain, were transformed with pER82-21. A single isolate was cultured in MJ minimal media (8) supplemented with 2,3-diketo-L-gulonate (DKG) in the presence of NADH, and the product of the reduction is believed to be 3-keto-L-gulonate (see Fig. 1A) (5, 6). DKG can be obtained from the hydrolysis of L-dehydroascorbate. Another operon in E. coli, yif-sga (now known as ula), is involved in the fermentation of L-ascorbate (5). The yiaK-S operon may be involved in the utilization of L-dehydroascorbate.

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‡ The abbreviations used are: DKG, 2,3-diketo-L-gulonate; JK loop, loop linking helices aJ and aK.

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with selenomethionine, lysine, phenylalanine, threonine, isoleucine, leucine, and valine for the production of selenomethionyl-labeled YiaK (9). Initial growth was carried out at 30 °C until the absorbance of the culture reached 1.0 unit. The incubation temperature was then decreased to 17 °C, and protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside at a final concentration of 1 mM. Following overnight incubation, the cells were harvested by centrifugation.

Selenomethionyl YiaK was purified by standard methods. Cell pellets were resuspended in lysis buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 10 mM imidazole, and 5 mM β-mercaptoethanol) and disrupted by sonication. The resulting lysate was clarified by centrifugation at 26,000 × g for 45 min at 4 °C. The supernatant was loaded onto a nickel-nitriilotriacetic acid column (Qiagen) and eluted in lysis buffer containing 250 mM imidazole. Fractions containing partially purified YiaK were pooled and loaded onto a gel filtration column (Superdex 75, Amersham Biosciences), and the running buffer contained 10 mM Tris (pH 8.0) and 5 mM dithiothreitol. The purified YiaK protein was concentrated to 10 mg/ml. Sample purity (>97%) and molecular mass (37.6 kDa) were verified by SDS-PAGE and laser desorption ionization-time-of-flight mass spectrometry, respectively. The yield of purified protein was ~16 mg/liter.

Crystalization of YiaK—The free enzyme of YiaK was crystallized at 298 K by the hanging drop vapor diffusion method. The reservoir solution contained 19% (w/v) polyethylene glycol 3350, 200 mM lithium sulfate, and 10 mM dithiothreitol. The crystals were transferred to paratone for cryoprotection and then flash-frozen in liquid propane for data collection at 100 K. The crystals belong to the space group P2₁, with cell parameters of a = 58.0 Å, b = 51.2 Å, c = 108.9 Å, and β = 105.8°. There are two molecules in the asymmetric unit.

Crystal Structure of YiaK in Complex with NAD and Tartrate—A crystal of YiaK complex with NAD was obtained with a reservoir solution containing 20% (w/v) polyethylene glycol 3350, 200 mM ammonium tartrate, and 20 mM NAD. The protein was pre-incubated with a 10 mM concentration of the substrate 2,3-diketogulonate (a kind gift from W. S. Yew and J. A. Gerlt). The crystals grew to full size in 3 days at 20 °C and were flash-frozen in liquid propane. They belong to the space group P1 with cell parameters of a = 75.5 Å, b = 81.3 Å, c = 113.2 Å, α = 79.6°, β = 77.2°, and γ = 82.0°. There are four dimers in the asymmetric unit.

**RESULTS AND DISCUSSION**

**Structure Determination**—The crystal structure of the free enzyme of E. coli YiaK (2,3-diketo-gulonate reductase) has been determined at 2.0 Å resolution by the selenomethionyl multiple wavelength anomalous diffraction method (15). The positions of the selenium atoms and the phases of the reflections were determined with the program SOLVE (11). This protein is unusually enriched with Met residues, with 15 such residues of 332 amino acids in each monomer. Five of these Met residues (Met77, Met157, Met177, Met251, and Met299) from each monomer are located in the dimer interface (Fig. 1B), giving rise to a close cluster of 10 Met residues. These residues are not conserved in other members of the family (Fig. 1B). The current atomic model for the free enzyme contains residues 1–332 for each monomer of the dimer, four sulfate ions, and 664 solvent water molecules. The model has low R values and excellent geometry (Table I).

The crystal structure of YiaK in complex with NAD and tartrate has been determined at 2.2 Å resolution by the molecular replacement method (Table I) (14). There are four dimers of the enzyme in this triclinic unit cell, and the eight monomers are named A through H, resulting in AB, CD, EF, and GH dimers. Two of these dimers (AB and GH) have NAD and tartrate in their active sites. For the other two dimers, the active site of one monomer in each (D and F) has a weak electron density for NAD but good density for tartrate, whereas the active site of the other monomer in each (C and E) is empty. The six monomers with occupied active sites have similar conformations, and the root mean square distance between equivalent Ca atoms of any pair of these monomers is about 0.4 Å. The two monomers (C and E) with empty active sites have conformations that are similar to the free enzyme (see below).

**Overall Structure of the YiaK Monomer, a New Backbone Fold**—The structure of the YiaK monomer contains 11 β-strands (named β1 through β11) and 12 α-helices (αA through αL) (Figs. 1B and 2A). The structure can be divided into approximately three domains, and this division is supported by the observation that the domains behave essentially as rigid bodies in the conformational transition that results from NAD(H) binding (see below). Domain I is made of residues from the N- and C-terminal segments of the protein (1–60, 318–332) and contains a four-helix bundle (Fig. 2A, αA, αE, αC, and αL). Strand β1, at the N terminus, and strand β11 form a small anti-parallel β-sheet that closes off one end of this bundle. The N-terminal initiator Met residue is part of strand β1 and has clear electron density.

Domain II is the central component of the structure. It contains a seven-stranded mostly anti-parallel β-sheet, which is covered on one face by two helices (αD and αE) (Fig. 2A). In comparison, most of the other face of this β-sheet is exposed in the structure of the monomer with the two helices on this face of the β-sheet (αH and αJ) located at the two ends of the β-sheet (Fig. 2B). The αJ helix has few interactions with the rest of the monomer, suggesting this conformation of the monomer is unlikely to be stable on its own (Fig. 2A). This exposed surface of the β-sheet is highly hydrophobic in nature (Fig. 2C), and it mediates the formation of the dimer. Dimerization also stabilizes the αJ helix (see below). At the C terminus of this domain, a long loop links helices αD and αE (Fig. 2A), and this JK loop is involved in binding the NAD cofactor as well as dimerization (see below).

Searches in the Protein Data Bank with the program DALI (16) did not show any other structures with a similar backbone fold to that of domain II. This suggests that this domain of YiaK may represent a new fold for a seven-stranded mostly anti-parallel β-sheet.
FIG. 1. Sequence alignment of *E. coli* YiaK and its homologs. A, chemical structures of the substrates and inhibitors of YiaK. B, representative sequences of the YiaK family from bacteria (*E. coli*), archaea (*Methanoccocus jannaschii*), and eukaryotes are aligned. The secondary structure elements in the crystal structure of YiaK are shown above the alignment. Strictly conserved and conservatively substituted residues are colored in red and blue. The diamonds below the sequences indicate residues that are involved in binding NAD (in green and black, the black ones are from the other monomer) or tartrate (magenta) or in dimerization (blue).
Domain III is an insertion on the surface of domain II, in the connection between strand β7 and helix αH (Fig. 2, A and B). This insertion covers residues 175–223, with two helices (αF and αG) and a two-stranded anti-parallel β-sheet (β8 and β9).

**A Tightly Associated Dimer**—Crystallographic analyses reveal that YiaK exists as dimers in the crystals, with an extensive interface (Fig. 2D). About 3500 Å² of the surface area of each monomer is buried at the dimer interface, suggesting that the dimers should be highly stable. The structural observations are corroborated by light scattering studies (data not shown), which show that YiaK exists as dimers in solution as well. Considering the facts that the conformation of the monomer is unlikely to be stable and that the NAD binding site is at the dimer interface (see below), it is likely that YiaK functions only as a dimer.

The extensive interface between the two monomers is mediated by the exposed face of their central β-sheet in domain II and the two associated helices (αH and αJ) (Fig. 2D). Most of the helix αH is buried in the core of the dimer, packing against the surface of the β-sheet in the other monomer. Helix αJ helps to cap off the two edges of this interface, by interacting with αH and αL. The beginning of the JK loop in one monomer is located next to the β5-β6 loop of the other monomer. In comparison, domain III and especially domain I are located distal from the dimer interface (Fig. 2D). Residues in this interface are generally conserved among the members of this family of enzymes (Fig. 1B), suggesting that they may all function as dimers.

**A Novel Mode of NAD Binding**—The NAD molecule in the AB and GH dimers has well defined electron density (Fig. 3A).
The molecule is associated mostly with one monomer, and several residues from domain II of the other monomer help define the binding site for the ADP portion of the cofactor (Fig. 3, B and C). The adenine base resides in a narrow groove formed by the side chains of Arg301 (in the JK loop), Trp147 (H56 loop), and Trp224 (H56 loop), showing amino-aromatic and π-π interactions (Fig. 3B). However, these residues are poorly conserved among the YiaK family members (Fig. 1B).

The ribose and the phosphates of the ADP portion of the cofactor are situated along strand H7 (residues 168–174) at the edge of the β-sheet in domain II (Figs. 2A and 3B). The 3'-hydroxyl group is hydrogen-bonded to the main chain carbonyl of residue 170 and the main chain amide of 172. One of the oxygen molecules on the β-phosphate makes a hydrogen bond with the main chain amide of residue 174. The β-phosphate group also shows ionic interaction with the highly conserved Lys225 residue (from H of the other monomer). The 2'-hydroxyl group of the ribose hydrogen bonds with the main chain amide of Gly304 (JK loop) and the side chain of Glu306 (αK), suggesting that YiaK may prefer NAD over NADP as the cofactor (see below).

The two hydroxyl groups on the ribose adjacent to the nicotinamide ring are hydrogen-bonded to the side chain of Asp172 (β7), which is conserved among the YiaK family members (Fig. 1B). The nicotinamide ring is placed over the side chain of the invariant Pro158 in strand β6. With this binding mode, a hydride transfer can only occur on the B face of the nicotinamide ring.

The observed interactions between NAD and YiaK appear to be unique to this family of enzymes. Many NAD(P)-dependent dehydrogenases contain a Rossmann fold that is for binding the dinucleotide cofactor (17), and the pyrophosphate group interacts with the GXGXX(G/A) fingerprint motif as well as the dipole of the helix immediately following this motif (7, 18). In contrast, the domain that binds NAD in YiaK (domain II) has a novel backbone fold, and the YiaK proteins do not contain the GXGXX(G/A) sequence motif. Instead, the pyrophosphate group interacts with a β-strand as well as the ammonium ion of a Lys residue from another monomer (Fig. 3B). Stabilization by interactions with the dipole of a helix is not observed in the YiaK-NAD complex either.

The 2'-hydroxyl group on the ribose next to the adenine base is hydrogen-bonded to Glu306 (αK helix) in YiaK (Fig. 3B). This interaction may be functionally equivalent to that from an Asp/Glu residue at the end of the β2 strand of the Rossmann folds (7, 19) and suggests that the YiaK family members with a conserved Glu residue at this position may only be able to use NAD as the cofactor. However, several members of the family have a different residue at this position, suggesting they could favor NADP as the cofactor.

The conformation of NAD in the YiaK complex is fully extended and is dramatically different from what is typically observed for NAD in complexes with enzymes containing a
Rossmann fold (Fig. 3D). With the nicotinamide-ribose portions of the cofactors in superposition, the adenine bases are separated from each other by ~8 Å in the two conformations (Fig. 3D). This conformational difference is because of a 140° rotation around the bond linking the α-phosphate and the bridging oxygen atom, as well as a rotation of 120° around the C-4’–C-5’ bond of the ribose. Interestingly, this NAD conformation has recently been observed in a complex with a mutant of nicotinamide-mononucleotide adenylyltransferase (20). The conformation of NAD in complex with the wild type of this enzyme is, however, entirely different (Fig. 3D). A large number of conformations have been observed for the NAD(P) cofactor in complex with various enzymes (19).

**Binding Mode of Tartrate**—We included 2,3-diketo-L-gulonate (Fig. 1A) in the crystallization solution at 20 mM concentration in an attempt to determine the binding mode of this substrate to the enzyme. To our surprise, crystallographic analyses based on the x-ray diffraction data show that a tartrate molecule is bound in the active site instead, with clearly defined electron density (Fig. 4A). Tartrate is present in the reservoir solution at 200 mM concentration, and this compound was required for obtaining good quality crystals of the enzyme in the presence of NAD. Tartrate is situated over the nicotinamide ring in the active site (Fig. 4B), and its hydroxyl and carboxylate groups are hydrogen bonded to several highly conserved residues of the enzyme (Fig. 4C). One of the oxygen atoms of the C-1 carboxylate makes two hydrogen bonds with the side chains of Arg48 and His116, whereas the other oxygen interacts with a solvent water molecule. His116 is strictly conserved among the YiaK family of enzymes, whereas Arg48 is mostly conserved (Fig. 1B). Arg48 also forms a hydrogen bond to the C-3 hydroxyl of tartrate (Fig. 4B). The C-4 hydroxyl of tartrate is hydrogen-bonded to the main chain amide nitrogens of Tyr180 and Gly181 at the beginning of helix αF, and it should also have favorable interactions with the dipole of this helix. The C-2 hydroxyl group of tartrate is hydrogen-bonded to the side chain of His44, which is the likely catalytic base of the enzyme (see below).

The structural information suggests that tartrate is likely an active site inhibitor of YiaK. Kinetic assays confirm that tartrate is a weak inhibitor of the enzyme. At 60 mM concentration this compound produced a 50% inhibition of the activity of YiaK. Interestingly, D-malate, which shares the same stereocchemistry for the hydroxyl group at the C-2 position as tartrate (Fig. 1A), can also inhibit the enzyme with similar potency. On the other hand, L-malate, with the opposite stereocchemistry (Fig. 1A), is a much poorer inhibitor of YiaK as compared with D-malate. None of these compounds can function as a substrate of the enzyme in the reduction of NAD to NADH.

**Conformational Changes upon NAD-Tartrate Binding Produce a More Open Active Site**—Structural comparisons reveal a large conformational change in YiaK upon the binding of NAD and tartrate. With domain II as the reference, both domain I and domain III show differences in their relative positions and orientations between the free enzyme and the NAD-tartrate complex (Fig. 5A). The largest conformational change is observed in domain III, which moves away from domain II, creating a larger opening near the active site for the binding of NAD (Fig. 5B). Within domain II, helices αH and αJ and the JK loop also show significant differences between the free enzyme and the NAD-tartrate complex (Fig. 5A). As these residues are involved in NAD binding as well as dimer formation, there is also a change in the organization of the dimer upon NAD-tartrate binding (not shown). With one monomer of the dimer in the superposition, a rotation of 7.5° is needed to bring the second monomer into overlap.

Interestingly, the conformational changes in YiaK occur in an unusual manner, as the active site region of the enzyme is more open in the complex with NAD and tartrate (Figs. 3C and 5, A and B). In comparison, most other enzymes assume a more...
closed conformation upon the binding of substrates and/or substrate-analog inhibitors, thereby shielding them from the solvent. In YiaK, however, one face of the tartrate molecule is completely exposed to the solvent (Fig. 4B). The nicotinamide ring of NAD is shielded from the solvent by tartrate, but the ADP portion has significant exposure to the solvent (Fig. 3C).

Such a conformational change of the enzyme is necessary for the binding of NAD and tartrate, as the structure of the free enzyme is incompatible with the presence of these molecules in the active site (Fig. 5C). Specifically, the side chains of His44 and Asp172 in the free enzyme are in steric clash with the nicotinamide-ribose portion of the cofactor, whereas residues Tyr180-Gly181 block the binding of the C-4 carboxylate group of tartrate.

In the crystal structure of the free enzyme, each monomer contains two sulfate ions in the active site. One of these sulfates superimposes with the β-phosphate of NAD, whereas the other sulfate is situated near the C-2 atom of tartrate (Fig. 5C). This second sulfate ion is hydrogen-bonded to the main chain amides of Tyr180 and Gly181, equivalent to the interactions from the C-4 carboxylate of tartrate. It is unlikely that the observed free enzyme conformation is stabilized by the binding of sulfate, because the two molecules in the crystal of the complex (monomers C and E) that have no NAD, tartrate, or sulfate bound to the active site assume conformations that are essentially the same as that observed in the free enzyme crystal.

Catalytic Mechanism of the Enzyme—In the structure of the NAD-tartrate complex, the C-2 carbon of tartrate is located 3.6 Å from the C-4 atom of the nicotinamide ring (Fig. 4C). At the same time, the hydroxyl group on the C-2 atom is hydrogen-bonded to the side chain of the strictly conserved His44 residue. Therefore, this binding mode may resemble that of the reduced product of the reaction. It is likely that His44 functions as the general base of the enzyme for the oxidation reaction. It extracts the proton from the hydroxyl group of the substrate, and this is coupled to the hydride transfer from the C-2 carbon to the cofactor NAD. For the reduction of DKG, this side chain in the protonated form could function as a general acid by polarizing the C-2 carbonyl and facilitating the hydride transfer from NADH to the substrate.

However, the stereochemistries at the C-2 positions of tartrate and 3-keto-L-gulonate are opposite of each other (Fig. 1A). It is not clear whether this compound can assume a similar binding mode as tartrate. It may have to be bound in the opposite orientation to maintain similar interactions with the enzyme. In this orientation, the carboxylate of gulonate could interact with the main chain amides of Tyr180 and Gly181, although a slightly more closed conformation of domain III may be needed for these interactions. It is possible that His116 (Fig. 4C), in addition to His44, may also have a role in extracting the proton from the substrate for catalysis. Attempts at producing crystals of the YiaK-DKG complex in the absence of tartrate have so far not been successful.

In summary, we have determined the crystal structures of the E. coli 2,3-diketo-L-gulonate reductase (YiaK), both alone and in complex with NAD and tartrate. The NAD molecule is bound to a domain with a novel polypeptide backbone fold, establishing the YiaK family of enzymes as a new class of
oxidoreductases. Structural and solution studies show that YiaK functions as a tightly associated dimer. Conformational changes in domains I and III of the enzyme are required for the binding of the NAD cofactor. In contrast to most other enzymes, these changes in YiaK produce a more open active site. Tartrate and the related D-malate are weak inhibitors of the enzyme. His^{44} is likely the catalytic residue of the enzyme.

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