YdiB and its paralog AroE are members of the quinate/shikimate 5-dehydrogenase family. Enzymes from this family function in the shikimate pathway that is essential for survival of microorganisms and plants and represent potential drug targets. Recent YdiB and AroE crystal structures revealed the presence of a NAD(P)-binding and a catalytic domain. We carried out site-directed mutagenesis of 8 putative active site residues in YdiB from *Escherichia coli* and analyzed structural and kinetic properties of the mutant enzymes. Our data indicate critical roles for an invariant lysine and aspartate residue in substrate binding and allowed us to differentiate between two previously proposed models for the binding of the substrate in the active site. Comparison of several YdiB and AroE structures led us to conclude that, upon cofactor binding and domain closure, the 2 identified binding residues are repositioned to bind to the substrate. Although the lysine residue contributes to some extent to the stabilization of the transition state, we did not identify any residue as catalytically essential. This indicates that catalysis does not operate through a general acid-base mechanism, as thought originally. Our improved understanding of the medically and agriculturally important quinate/shikimate 5-dehydrogenase family at the molecular level may prove useful in the development of novel herbicides and antimicrobial agents.

The shikimate pathway of prokaryotes, fungi, plants, and apicomplexa is essential for survival. The main route of this pathway leads to the biosynthesis of chorismate, the precursor of essential aromatic compounds including vitamins and amino acids (Fig. 1) (1, 2). The successful targeting of the shikimate pathway in crop plants by glyphosate has spurred further efforts in herbicide development (3). More recently, the occurrence of herbicide-resistant weeds (4) has led to the development of transgenic crops with increased glyphosate tolerance (5, 6). In bacteria, the shikimate pathway has been subject to metabolic engineering, aimed at the possible industrial production of high value hydroaromatic compounds (7). Lastly, their absence in metazoans makes the enzymes of the shikimate pathway potential targets for novel antimicrobial agents (3, 8–10). With a view to the design of inhibitors of the shikimate pathway, the understanding at the molecular level of enzymes involved in this pathway has received much attention over the last 25 years (3, 11).

Step 4 of the pathway, the reversible NADPH-dependent reduction of 3-dehydroshikimate to shikimate (Fig. 2) is catalyzed by the enzyme shikimate dehydrogenase (EC 1.1.1.25), a member of the quinate/shikimate 5-dehydrogenase family. In addition to the widely distributed bacterial NADP-dependent shikimate dehydrogenase AroE, *Escherichia coli*, *Salmonella typhimurium*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* also possess a paralogous enzyme, YdiB. YdiB from *E. coli* is a dual specificity quinate/shikimate NAD-dependent dehydrogenase, and its possible evolution and metabolic role have been discussed recently (12). Although the involvement of AroE in the shikimate pathway is well established for *E. coli* and *S. typhimurium* (13), the actual biological function of YdiB remains unclear, nor is it known whether 3-dehydroshikimate or quinate represents the natural substrate of YdiB. Nevertheless, the YdiB enzymes from *S. pneumoniae* and *H. influenzae* were recently shown to be essential for *in vitro* growth of these human pathogens (14). A similar occurrence of two family members is also found in filamentous fungi. In addition to a NADP-dependent shikimate dehydrogenase, they possess a related enzyme, quinate dehydrogenase (EC 1.1.1.24), that catalyzes the NAD-dependent oxidation of quinate. This is the first step of the quinate pathway (Fig. 1), which affords growth on quinate as a carbon source (15).

In fungi (16) and the apicomplexan parasite *Toxoplasma gondii* (17), the NADP-dependent shikimate dehydrogenase constitutes the C-terminal module of a penta-functional polypeptide, which combines the enzymatic activities for steps 2–6 of the shikimate pathway (Fig. 1). In plants (18) and bacteria from the genus *Chlamydia*, this enzyme forms the C-terminal module of a bifunctional polypeptide catalyzing steps 3 and 4. Most bacterial shikimate dehydrogenases, and the fungal quinate-oxidizing dehydrogenases, on the other hand, represent monofunctional enzymes of 29–36 kDa. Few studies have been performed on the kinetic or chemical mechanism of catalysis for this family of enzymes. For the shikimate dehydrogenase from *Pisum sativum*, it has been shown that the kinetic mechanism is ordered Bi Bi in both directions, with the cofactor adding first (19). As additionally demonstrated for the shikimate dehydrogenase from *E. coli* (20), the mechanism involves the stereoselective transfer of hydrogen between the A side of NADPH and the substrate (19). Recently, the crystal structures of AroE from *E. coli*, Meth-
anococcus jannaschii, and H. influenzae (12, 21, 22) and of YdiB from E. coli (12, 23) were solved in complex with NADP and NAD, respectively. Structures of apoenzymes are also available for H. influenzae AroE (22) and YdiB. All of these structures reveal a common fold comprising two domains separated by a cleft. Although AroE from E. coli and AroE and YdiB from H. influenzae exist as monomers (12, 22), YdiB from E. coli (12, 23) and AroE from M. jannaschii (21) both homodimerize via their N-terminal domains. This portion of the protein possesses a unique α-β-α sandwich motif, which also includes an α-helical hairpin structure at the C terminus of the protein and is further referred to as domain 1. The intervening sequence, domain 2, forms a Rossmann fold, to which the dinucleotide cofactor is bound with the A side of the nicotinamide ring facing the interdomain cleft (12, 21, 23). A direct comparison of the cofactor complexes for the two E. coli enzymes AroE and YdiB by Michel et al. (12) revealed structural differences in their nucleotide-binding motifs, which likely account for the 10-fold higher affinity of YdiB for NAD than for NADP. In contrast to E. coli YdiB, AroE enzymes are generally NADP-specific.

Despite the recent availability of several crystal structures for enzyme-cofactor complexes, as well as for apoenzymes of quinate/ shikimate 5-dehydrogenases, no structural data on substrate binding are yet available, and so the nature of the active site in...
this agriculturally and medically important enzyme family has remained ambiguous. The active site is identified by the position of the nicotinamide moiety and appears to be defined by an invariant aspartate and lysine residue in substrate binding. Surprisingly, none of the mutated residues was essential for catalysis, suggesting that the primary catalytic mechanism does not involve general acid-base catalysis.

EXPERIMENTAL PROCEDURES

Preparation and Characterization of YdiB Mutants—Site-directed mutagenesis was performed on the plasmid containing the ydiB gene (12) using the QuickChange™ XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). Forward versions of the mutagenic primers are listed in Table I. The sequences of the mutant ydiB genes were confirmed by DNA sequencing. YdiB enzymes were overexpressed and purified as described previously for wild-type protein (12). Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories) based on the original Bradford assay (24) with bovine serum albumin as the standard. Mass spectra were recorded using an Agilent 1100 Series liquid chromatograph/mass selective detector instrument (Agilent Technologies, Mississauga, Ontario, Canada) in electrospray ionization mode and analyzed using Agilent ChemStation software (version A.09.01). A sample of purified YdiB protein (0.1–0.2 mg/ml) was diluted 1:100 (v/v) in 10% (v/v) glycerol, 3 mM NADH. Kinetic data were evaluated by non-linear regression.

Initial velocities of enzyme activity were determined by one-in-one dilutions (v/v) of reduced cofactor in a volume of 210 μl/well, starting from 0.4 mM NADH. Kinetic data were evaluated by non-linear regression with the Michaelis-Menten equation (v = V_{max} [S]/(K_{M} + [S]), using the SigmaPlot software (SPSS Science, Chicago, IL). The catalytic constant, k_{cat}, was calculated using the equation V_{max} = k_{cat} × [E], where [E] = total enzyme concentration.

RESULTS AND DISCUSSION

Structural Characterization of YdiB Mutants—The structure of E. coli YdiB has been determined independently by two groups (12, 23). Although one of the E. coli YdiB models (PDB3 number 1NPD) includes all 288 residues of the protein (23), the other (PDB number 1O9B) includes residues 7–286 (12). In the current work, we made use of the same YdiB construct and YdiB purification protocol as described in the latter report (12). To determine whether the purified enzyme was truncated, we analyzed the wild-type protein by mass spectrometry. Liquid chromatograph/mass selective detector analysis gave a mass for YdiB of 31,361 Da, as compared with a calculated mass of 31,371 Da, which agrees within the expected mass error of about 0.02%. This mass includes the additional Gly-Ser residues that remain at the N terminus after thrombin cleavage with protein expressed using the pGEX-4T1 vector (12). In the following, we will refer to residues according to E. coli YdiB numbering. Where specified otherwise, E. coli YdiB numbering will be referenced in parentheses.

Sequence alignments of quinate/shikimate 5-dehydrogenase family members from Pfam (27) and the NCBI Conserved Domain Database4 (28) indicate that the residues Gly^{255}, Ser/Thr^{256}, Ser/Thr^{67}, Tyr^{99}, Asn^{92}, Asp^{107}, Gly^{131}, Gly^{133}, Gly^{134}, Gly^{255}, and Gln^{262} are likely fully conserved. In addition, Asn^{105} and Arg^{156} are also conserved in nearly all sequences. The cluster of glycines (at positions 131, 133, and 134) is part of the P-loop that interacts with the phosphates of the NAD(P) cofactor, and the carbonyl group of Gly^{255} hydrogen-bonds to the amide group of the nicotinamide ring. We have concentrated on the highly conserved residues Ser^{67}, Lys^{71}, Asn^{92}, Asp^{107}, and Gln^{262} in the vicinity of the putative substrate-binding site as the most likely candidates for residues essential for catalysis. Additionally, we selected the partially conserved residues Ser^{22}, Tyr^{29}, and Thr^{106} as potential substrate-binding residues (Table I, Fig. 3). We have not attempted to mutate Arg^{156} as it is remote from the catalytic site and stacks with the adenine ring of NAD. Similarly, Asn^{105} appears to be shielded from the active site by Asp^{107} and likely plays a structural role. The mutants S22A, Y39F, S67A, K71G, N92A, T106A, D107A, and Q262A were expressed and, with the exception of N92A, purified to apparent homogeneity as assessed by SDS-PAGE (not shown). The N92A mutant protein appeared more sensitive to proteolytic cleavage than wild-type YdiB or any of the other mutants and was not isolated in a pure form.

To assess the effects of the various mutations on YdiB structure, we characterized the conformational integrity of the purified proteins using several methods. Native PAGE showed well focused bands for the wild-type protein, as well as for the S22A, K71G, Q262A, Y39F, and S67A mutants, and less-focused bands for the D107A and T106A mutants (Fig. 4A). The
N92A mutant appeared as a smear. Dynamic light scattering measurements on the various purified proteins were mostly in agreement with the results from native PAGE, with the N92A mutant being polydisperse and yielding an apparent molecular mass of 300 kDa (not shown). Consistent with the results from native PAGE, the general shape of the CD spectra for wild-type enzyme and the S67A and K71G mutants were similar, whereas the alanine mutations of Thr106 and Asp107 caused apparent shifts (Fig. 4B). The N92A mutant exhibited a spectrum that deviated significantly from either wild-type YdiB or the other YdiB mutants. In analytical gel filtration experiments, wild-type YdiB exhibited an apparent mass of ~57 kDa (calculated mass for the recombinant dimer: 62.7 kDa). In addition, the wild-type contained a very small amount (0.2%) of a species with an apparent mass of ~33 kDa (likely monomeric form) and some protein that eluted in the void volume of the column. About 65% of the N92A mutant protein appeared in the void volume and was likely aggregated, with the remainder eluting at about 33 kDa. Although the T106A mutant also showed considerable amounts of apparent aggregate (12%) and a 33-kDa species (1.8%), it largely eluted as a 57-kDa species (likely dimeric form). The D107A profile was most similar to that of the wild type.

Taken together, these data suggested that the N92A protein was improperly folded. The protein was partly degraded, likely by trace amounts of proteolytic enzymes. We assume that the fragments aggregated into larger species. The Asn92 residue is located at a -bulge, at the N-terminal end of strand 4 within the 6-stranded mixed -sheet of domain 1 (Fig. 3), and participates in a hydrogen-bonding network through its side chain and main chain atoms (12), thereby offering an explanation for the apparent effect of this mutation on the YdiB structure. Mutation of either Asn92 or Thr106 to alanine would disrupt the H-bond between Asn92(OD1) and Thr106(OG). The side chain of Thr106 also forms a hydrogen bond with Gln262, helping to orient both side chains in the active site. Thr106 and Asp107 are

| Mutant | Oligonucleotide sequencea | Degree of residue conservationb |
|--------|---------------------------|-------------------------------|
| S22A   | 5’-TATCCGCCACAGTTCTGCACGAG-3’ | 76% |
| Y39F   | 5’-GATCAAGGCTGATGAAATGCGCCAGGAT-3’ | 85% |
| S67A   | 5’-ATGCGGCGACATCCGAAAGCTGATCAG-3’ | 21% |
| K71G   | 5’-CTGCGATTGCGCGACACGATGCTCAG-3’ | 100% |
| N92A   | 5’-GTTGCCACACTGCGGCGACGACCTGATC-3’ | 99% |
| T106A  | 5’-CTGCGATTGCGCGACACGATGCTCAG-3’ | 99% |
| D107A  | 5’-CTGCGATTGCGCGACACGATGCTCAG-3’ | 100% |
| Q262A  | 5’-ATGCGGCGACATCCGAAAGCTGATCAG-3’ | 99% |

a Altered nucleotides, that introduced the desired mutations, are underlined. Additional silent mutations, introducing restriction enzyme cleavage sites for screening purposes, are printed in italics.

b The degree of conservation for the mutated residue in E. coli YdiB among ~75 members of the quinate/shikimate 5-dehydrogenase family, as aligned in the NCBI Conserved Domain Database (28), is indicated.

c Threonine, not serine is the prevailing amino acid at position 67, with a conservation level of 40%.
The mutants K71G, T106A, and D107A showed marked reductions in catalytic efficiencies in the substrate-dependent kinetics (Table II). As compared with the wild-type enzyme, the catalytic efficiency of NAD reduction by these mutants with shikimate was reduced by 3, 2, and nearly 4 orders of magnitude, respectively. For K71G and T106A, at least a 10-fold greater reduction in catalytic efficiency was observed with quinate than with shikimate. No activity toward quinate could be detected for the D107A mutant. The loss of activity in K71G, T106A, and D107A was predominantly due to significantly increased $K_M$ values for the substrate, with much smaller effects on $k_{cat}$ values. Indeed, the $k_{cat}$ values determined from substrate-dependent kinetics for the T106A and D107A mutants remained practically unchanged, whereas those for the K71G mutant were reduced ~9-fold. For the latter mutant, a significant decrease in $K_M$ for the NAD cofactor was observed (higher affinity), which offset the parallel decrease in $k_{cat}$ values, leading to a continued efficient catalytic reduction of NAD. By comparison, no pronounced changes in the measured cofactor kinetics were observed for the T106A and D107A mutants. We note that we could not saturate the K71G and T106A mutants with quinate and could not saturate the D107A mutant with shikimate during the cofactor kinetics due to high $K_M$ values for the respective substrates (Table II). Therefore, the catalytic constants for the cofactor are somewhat underestimated in these cases. We further suggest that the lack of detectable activity for the D107A mutant with quinate reflects the highest loss of substrate affinity.

We cannot rule out that steps other than the chemical transformation, i.e. the hydride transfer reaction, affected the apparent $k_{cat}$ values. The measured differences in kinetic parameters could also be affected by structural perturbation in some of the mutant enzymes, as discussed above. Nevertheless, our data (Table II) indicate important roles for Lys71 and Asp107 in substrate binding in the Michaelis complex. The general decrease in $k_{cat}$ for the structurally intact K71G mutant likely reflects a contribution of this residue to stabilization of the transition state. Importantly, none of the mutated residues was found to be essential for catalysis.

**Active Site Structures of YdiB and AroE Enzymes**—The sequence identity of E. coli YdiB with related proteins of known structure ranges from 23% (H. influenzae AroE) to 36% (M. jannaschii AroE). The overall structure of E. coli YdiB comprises two domains (23), which are each similar to the structures of the corresponding domains of H. influenzae YdiB1 and of AroE from E. coli (12), H. influenzae (22), and M. jannaschii (21). However, as was shown previously based on the superposition of four independent molecules of AroE and two molecules of YdiB (12), the individual molecules display conformational variability, characterized by a ~25° rotation of one domain relative to the other. Domain movements of similar magnitude can also be observed in the YdiB structure determined by Benach et al. (23).

For spatial comparison of the substrate-binding site, we considered superposition of the individual chains using the putative active site residues of domain 1. Among the 16 individual chains compared (Table III), for 6 of the residues mutated in this study, Ser22, Tyr39, Ser67, Lys71, Asp92, and Thr106, the main and side chain atoms superimpose very well. As an exception, Ser67 (Ser47) in the four chains of the H. influenzae YdiB structure differs in the orientation of the side chain by ~90° ($\chi_1$). Among the 16 chains, the side chains of Gln262 cluster in two different orientations. Although an orientation toward the side chain of Gln262 predominates, the side chain faces the opposite direction in chain A of H. influenzae YdiB and in both chain A and chain B from H. influenzae AroE.

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one molecule of these apoenzyme structures each, this reorientation is associated with the presence of a hydrogen bond between Gln262 and Asp107 (Table III). Furthermore, a salt bridge is formed between Lys71 and Asp107 in 5 out of 10 enzyme cofactor complexes without major conformational changes as compared with the apo-enzyme structures.

When the NAD(P)-binding domains are superimposed independently, the cofactors in various structures cluster closely together. Relative to domain 1, however, the disposition of NAD(P) molecules shows a broader distribution that results primarily from domain flexibility (12). This is most pronounced for the adenine dinucleotide portion of NAD. In contrast, conformational changes associated with substrate-enzyme interactions (12). This is most pronounced for the adenine dinucleotide portion of NAD. In contrast, conformational changes associated with substrate-enzyme interactions (12). This is most pronounced for the adenine dinucleotide portion of NAD. In contrast, conformational changes associated with substrate-enzyme interactions (12). This is most pronounced for the adenine dinucleotide portion of NAD. In contrast, conformational changes associated with substrate-enzyme interactions (12). This is most pronounced for the adenine dinucleotide portion of NAD. 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The approximate orientation of the substrate relative to neighboring protein side chains is projected. Proposed protein-substrate interactions in the ternary complex are highlighted by gray background shading. In model A, the C-4 and C-3 hydroxyl of the substrate are coordinated by Lys\textsuperscript{71}/Asp\textsuperscript{107} and Gln\textsuperscript{262}, respectively, whereas Ser\textsuperscript{20}, Ser\textsuperscript{22}, and Tyr\textsuperscript{234} hydrogen-bond to the C-1 carboxylate. In model B, the C-5, C-4, and C-3 hydroxyls hydrogen-bond to Asp\textsuperscript{107}, Gln\textsuperscript{262}, and Tyr\textsuperscript{234}, respectively. The results from this study support model A.

Within our comparison set of quinate/shikimate 5-dehydrogenase molecules (Table III), the Asp\textsuperscript{107}-Gln\textsuperscript{262} hydrogen bond and the Lys\textsuperscript{71}-Asp\textsuperscript{107} salt bridge are mutually exclusive. The former is observed predominantly in the structures of apoenzymes, whereas the latter is only found in enzyme-cofactor complexes, which were found to adopt more closed conformations. The breaking of the Asp\textsuperscript{107}-Gln\textsuperscript{262} hydrogen bond and the formation of Lys\textsuperscript{71}-Asp\textsuperscript{107} salt bridge, therefore, may be associated with active site closure after cofactor binding and the formation of a productive catalytic site, as proposed previously (12).

Substrate Binding and Catalysis—To assign functional roles to putative active site residues in the quinate/shikimate 5-dehydrogenase family, three models for ternary enzyme-cofactor-substrate complexes have been proposed previously, representing two essentially different possible orientations of the substrate in the active site (Fig. 5). Based on the crystal structures of enzyme-cofactor complexes, Michel et al. (12) modeled 3-dehydroshikimate into the E. coli AroE structure (Model A), whereas Benach et al. (23) modeled shikimate into the E. coli YdiB structure (Model B). The third model, proposed by Ye et al. (22), shows 3-dehydroshikimate bound to H. influenzae AroE. As far as we can deduce from the report (22), the orientation of the substrate is similar to the model of Benach et al. (23).

Our structure-function analysis included 5 of 7 putative substrate-interacting residues from models A and B (Fig. 5) together. We observed a clear decrease in substrate affinity only for the K71G and D107A mutants (Table II). Previous studies demonstrated a high importance of the C-4 hydroxyl over the C-3 hydroxyl (Fig. 2, shikimate numbering) in the binding of shikimate and 3-dehydroshikimate to the shikimate dehydrogenase from P. sativum (29) and of shikimate to E. coli AroE (30).

An increased distance of 4.4 Å for chain C of the E. coli AroE structure is indicative of a more open enzyme active site, as proposed previously (12). This interaction may persist during the hydride transfer reaction, with only Lys\textsuperscript{71} contributing appreciably to transition state stabilization as already mentioned. YdiB was previously suggested to operate against the background of our structural comparison and mutational analysis, we propose that, upon cofactor binding and domain closure, the 2 fully conserved residues Lys\textsuperscript{71} and Asp\textsuperscript{107} form a salt bridge, which orients them for the initial binding of the substrate, i.e. 3-dehydroshikimate or shikimate, as proposed by Model A (Fig. 5) (12). This interaction may persist during the hydride transfer reaction, with only Lys\textsuperscript{71} contributing appreciably to transition state stabilization as already mentioned. YdiB was previously suggested to operate by general acid-base catalysis (12, 23). During substrate reduction (Fig. 2), the enzyme would stabilize the accumulating negative charge at the hydride-accepting C-3 carbonyl oxygen of the substrate by a concerted proton transfer reaction (23). Our kinetic analysis, however, did not identify any residue as catalytically essential, one that would represent a potential acid-base catalyst.

The retention of high catalytic rates for a NADP(H)-dependent reductase in the absence of general acid-base catalysis is not
unprecedented. After mutation of the primary acid-base catalyst Tyr$^{25}$ in 3α-hydroxysteroid dehydrogenase, Penning and co-workers (32) reported wild-type-like $k_{cat}$ values for quinone reduction via a mechanism different from 3-ketosteroid reduction by the native enzyme and most likely facilitated by a proximate effect. In addition to the classical models of barrier crossing in enzyme reactions, the work of Klinam and co-workers (39) demonstrated the relevance of quantum mechanical tunneling over a wide range of physiological temperatures as another route (reviewed in Ref. 33). Domain flexibility in liver alcohol dehydrogenase was recently substantiated by Plapp and colleagues (36, 37), who implicated the nicotinamide-binding site in the energetics of the conformational change in addition to its proposed role in cofactor activation (38). The significance of such properties for catalysis in YdiB remains speculative.

In conclusion, the results from this study do not support a role for general acid-base catalysis for *E. coli* YdiB. To elucidate the catalytic pathway, structural information for the ternary enzyme-cofactor-substrate complex and more detailed kinetic analyses of enzyme mutants are needed. Our data suggest primary roles for the invariant residues Lys$^{71}$ and Asp$^{197}$ in substrate binding, which is most consistent with a specific role for general acid-base catalysis for conformational change in addition to its proposed role in cofactor activation (38). The significance of such properties for catalysis in YdiB remains speculative.

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