A New View of the Mechanisms of UDP-N-Acetylglucosamine Enolpyruvyl Transferase (MurA) and 5-Enolpyruvylshikimate-3-phosphate Synthase (AroA) Derived from X-ray Structures of Their Tetrahedral Reaction Intermediate States*

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Received for publication, September 3, 2003, and in revised form, September 15, 2003
Published, JBC Papers in Press, September 16, 2003, DOI 10.1074/jbc.M309741200

UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) and 5-enolpyruvylshikimate-3-phosphate synthase (AroA) constitute the small enzyme family of enolpyruvyl transferases, which catalyze the chemically unusual reaction of enolpyruvyl transfer. MurA catalyzes the first step in the biosynthesis of the bacterial cell wall; AroA is the sixth enzyme of the shikimate pathway leading to the synthesis of aromatic compounds in numerous microorganisms and plants. Because both metabolic pathways are absent from mammals but essential for the growth of microorganisms, MurA and AroA are attractive targets for the development of novel antimicrobial drugs. We have determined the x-ray structures of the D305A mutant of Entero bacter cloacae MurA and the D313A mutant of Escherichia coli AroA, both of which crystallized in the presence of their substrates. The structures depict the tetrahedral reaction intermediate states of the enzymes and prove that, without the aspartate side chain, the overall addition-elimination reaction in both enzymes is halted after the addition step. The presented structures lead to a new view of the catalytic mechanism and, moreover, provide an ideal starting point for the rational design of potent inhibitors of MurA and AroA.

* This work was supported by the National Institutes of Health Center of Biomedical Research Excellence, the Kansas National Science Foundation Experimental Program to Stimulate Competitive Research (NSF EPSCoR), and the General Research Fund of the University of Kansas. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: MurA, UDP-N-acetylglucosamine enolpyruvyl transferase; AroA, 5-enolpyruvyl shikimate-3-phosphate synthase; PEP, phosphoenolpyruvate; UNAG, UDP-N-acetylglucosamine; S3P, shikimate 3-phosphate; MES, 2-[N-morpholino]ethanesulfonic acid; THI, tetrahedral intermediate.

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UDP-N-acetylglucosamine enolpyruvyl transferase (MurA; EC 2.5.1.17) and 5-enolpyruvylshikimate-3-phosphate synthase (AroA; EC 2.5.1.19) constitute the small enzyme family of enolpyruvyl transferases, which catalyze the chemically unusual reaction of enolpyruvyl transfer. MurA catalyzes the first step in the biosynthesis of the bacterial cell wall; AroA is the sixth enzyme of the shikimate pathway leading to the synthesis of aromatic compounds in numerous microorganisms and plants. Because both metabolic pathways are absent from mammals but essential for the growth of microorganisms, MurA and AroA are attractive targets for the development of novel antimicrobial drugs. We have determined the x-ray structures of the D305A mutant of Entero bacter cloacae MurA and the D313A mutant of Escherichia coli AroA, both of which crystallized in the presence of their substrates. The structures depict the tetrahedral reaction intermediate states of the enzymes and prove that, without the aspartate side chain, the overall addition-elimination reaction in both enzymes is halted after the addition step. The presented structures lead to a new view of the catalytic mechanism and, moreover, provide an ideal starting point for the rational design of potent inhibitors of MurA and AroA.
Analysis of the vinyl ether products of MurA and AroA generated from stereospecifically isotope-labeled PEP has shown that the addition and elimination steps proceed with opposite stereochemistry (22–24). If the stereochemistry of the addition of the first bound substrate to C-2 of PEP and that of a proton to C-3 of PEP is anti, the elimination of H/H11001 from C-3 and Pi from C-2 of the tetrahedral reaction intermediate has to be syn, or vice versa.

Despite the detailed knowledge of the active sites of both enolpyruvyl transferases, a precise comparison was not possible in the past because only a limited number of active site residues are conserved between MurA and AroA, and, moreover, some of these residues differ significantly in their spatial arrangement. In our attempt to trap reaction intermediate states of enolpyruvyl transferases, we substituted alanine for a strictly conserved aspartic acid (Asp-305 for MurA and Asp-313 for AroA) in the active site of the two enzymes and co-crystallized these mutant enzymes with substrates. Subsequent structure determination to resolutions of 2.65 and 1.6 Å revealed the tetrahedral reaction intermediate states of MurA and AroA, respectively. The chiral center at C-2 of the PEP moiety of the observed tetrahedral intermediates now gives a new reference point for the alignment of the two structures, such that different regions of the active sites of the two enzymes can be mapped according to their function in the enolpyruvyl transfer reaction. Here, we discuss the implications of the new structures for the catalytic mechanism and rational inhibitor design.

**EXPERIMENTAL PROCEDURES**

S3P (barium salt) was a gift from Nikolaus Amrhein (ETH, Zürich, Switzerland). PEP (potassium salt) and UNAG were purchased from Sigma. Protein concentration was determined using the Pierce Coomassie reagent with bovine serum albumin as standard.

The mutations D305A and D313A were introduced to wild-type Enterobacter cloacae MurA and wild-type E. coli AroA, respectively, using the QuikChange mutagenesis kit from Stratagene and appropriate primers. pET vectors 9d and 24d (Novagen) containing the open reading...
frame of the wild-type enzymes were used as templates for the point-mutation of MurA and AroA, respectively. D305A-MurA was overexpressed in BL21(DE3)-competent (Stratagene) E. coli cells, and D313A-AroA was overexpressed in STBL2(DE3) E. coli cells (20). The overexpressed proteins were purified as described (17, 20).

The mutant enzymes were concentrated to ~100 mg/ml using Centricon 30 devices (Amicon) at 4 °C. D305A-MurA was crystallized at 19 °C from 10 mM MES (pH 6.4), 10% (w/v) polyethylene glycol 20,000 in the presence of 5 mM UNAG and 5 mM PEP. D313A-AroA was crystallized at 19 °C from 4 mM sodium formate in the presence of 5 mM S3P and 5 mM PEP.

Diffraction data were recorded at ~180 °C using the rotation method on single flash-frozen crystals of D305A-MurA (detector, a R-axis IV* imaging plate detector from MSC; The Woodlands, TX; x-rays, CuKα, focused by mirror optics, from MSC; and the generator, Rigaku RU300 from MSC) and D313A-AroA (detector, Mar 345 mm imaging plate detector from X-ray Research, Norderstedt, Germany; x-rays, CuKα, focused by mirror optics, from MSC; and the generator, Rigaku RU300 from MSC). The MurA data were reduced with HKL suite (25) and the AroA data with XDS (26). For phasing and refinement, the program package CNS (27) was used, and model building was performed with O (28).

The structures have been solved by molecular replacement. For li-
ganded D313A-AroA, the structure of the binary AroA:S3P complex (19) (Protein Data Bank entry code 1G6S), omitting solvent molecules, ions, and ligands, could readily be used as starting model. A tetramer of MurA molecules derived from another oligomeric crystal form of ligan-
ded MurA* stripped of solvent molecules and ligands served as search model for liganded D305A-MurA. In the cross-rotation and translation search, diffraction data have been limited to low-resolution reflections 20.0–6.0 Å. From the 30 highest peaks of the cross-rotation function, four solutions could be successfully translated into the asymmetric unit of the crystal, revealing that the asymmetric unit contains four tetramers of MurA molecules. Independent rigid body refinement of the 16 MurA molecules produced four solutions, which, in the following discussion, will be responsible for PEP binding and enolpyruvyl transfer. Notably, all binding partners of the PEP moiety of the intermediate state are identical with those of the herbicide glyphosate in AroA (19), corroborating earlier suggestions that glyphosate and PEP target the same binding site and that the AroA:S3P-glyphosate complex indeed mimics an intermediate state of the AroA reaction (12, 19, 32). Although the charged residues listed above execute principally identical interactions with the PEP moiety of the tetrahedral adducts and even adopt similar side-chain conformations in MurA and AroA, the PEP binding site is significantly distorted between the two enolpyruvyl transferases. The distortion becomes obvious by superimposing the two enzyme structures with the chiral centers of the PEP C-2 atom of the tetrahedral adducts as reference (Fig. 5, bottom). Although residue 305/313 and the guanidinium

Now possible to superimpose both active sites precisely. The MurA:THI and AroA:THI complexes reveal a set of strictly conserved charged residues that interact with the PEP moiety of the tetrahedral adduct (Figs. 4 and 5). These are residues Lys-129, Arg-120, Arg-331, and Arg-371 in MurA, which corre-
spond to Lys-22, Arg-124, Arg-344, and Arg-386, respectively, in AroA. The charged side chains of these residues appear to be responsible for PEP binding and enolpyruvyl transfer. Notably, all binding partners of the PEP moiety of the intermediate state are identical with those of the herbicide glyphosate in AroA (19), corroborating earlier suggestions that glyphosate and PEP target the same binding site and that the AroA:S3P-glyphosate complex indeed mimics an intermediate state of the AroA reaction (12, 19, 32). Although the charged residues listed above execute principally identical interactions with the PEP moiety of the tetrahedral adducts and even adopt similar side-chain conformations in MurA and AroA, the PEP binding site is significantly distorted between the two enolpyruvyl transferases. The distortion becomes obvious by superimposing the two enzyme structures with the chiral centers of the PEP C-2 atom of the tetrahedral adducts as reference (Fig. 5, bottom). Although residue 305/313 and the guanidinium group of Arg-371/Arg-386 overlap within the coordinate error, Lys-22, Arg-120/Arg-124, and Arg-331/Arg-344 are set-off by up to 2.0 Å.

In addition to these geometrical deviations, the PEP binding sites of MurA and AroA differ in four residues, which exclu-
sively occur in AroA or MurA. Arg-397, which is strictly con-
served in MurA, corresponds to Lys-411 in AroA. Because this replacement is conservative in charge and side-chain length, the salt-bridge to the phosphate group of the PEP-moiety is preserved. A more radical difference is the existence of Glu-341 of AroA versus Cys-115 of MurA, which have been implicated

### Table I

| Data set       | D313A-AroA   | D305A-MurA   |
|----------------|--------------|--------------|
| Space group    | P2₁2₁2₁     | P₂        |
| Unit cell dimensions | a (Å) | 57.8 | 139.5 |
|                 | b (Å) | 85.1 | 153.9 |
|                 | c (Å) | 87.5 | 167.5 |
| α (°)          | 90     | 90    |
| β (°)          | 90     | 112.95 |
| γ(°)           | 90     | 90    |
| Molecules/asymmetric unit | 1     | 16    |
| Protein atoms | 3229   | 50240   |
| Alternate atom positions | 39 | 0 |
| Ligand atoms | 26     | 784    |
| Solvent molecules | 543 | 1431   |
| Formate ions  | 8      | 0      |
| Ethylene glycol molecules | 0 | 16    |
| R.m.s.d. bonds (Å) | 0.016  | 0.007 |
| R.m.s.d. angles (°) | 1.79  | 1.25   |
| Resolution range (Å) | 15–1.6 | 20–2.85 |
| (last shell) | (1.65–1.60 Å) | (2.70–2.85 Å) |
| Measured reflections | 3059999 | 809759 |
| Completeness (%) | 97.95 | 98.95 |
| Rsym (%)      | 5.9 (33) | 9.8 (33.5) |
| Rfree (%)     | 15.5 (26) | 21.6 |
| Rfree (%)     | 18.7 | 25.3    |

* a R.m.s.d. is root mean square deviation from ideal values.
  * b Rsym = 100 × ∑ |Fobs − Fmodel| / ∑ FobsWhere Fobs and Fmodel are observed and calculated structure factor amplitudes, respectively.
  * c Rfree = Rfree calculated for randomly chosen reflections, which were excluded from the refinement (3% and 2% of the unique reflections of D313A-AroA and D305A-MurA, respectively).

### RESULTS AND DISCUSSION

The mutant enzymes D305A-MurA from E. cloacae and D313A-AroA from E. coli have been co-crystallized with their substrates, UNAG and S3P, respectively, and PEP. Subsequent structure determination to resolutions of 2.65 (MurA) and 1.6 Å (AroA) revealed the tetrahedral reaction intermediate states of the enzymes (Figs. 3, 4, and 5), which, in the following discussion, are denoted MurA:THI and AroA:THI, respectively. Both enzymes are in the closed conformation observed previously for liganded MurA (11, 15) and liganded AroA (19) (Fig. 3). The root mean square deviation is 0.6 Å between 418 equivalent pairs of Ca atoms of E. coli C115A-MurA liganded with a fluorinated tetrahedral intermediate analog (Protein Data Bank entry code 1A2N; Ref. 15) and of MurA:THI, where the largest differences occur in the backbone around residues 82–88 and residues 112–119. Comparing AroA:THI with the structure of the binary AroA:S3P complex (Protein Data Bank entry code 1G6T; Ref. 19), the root mean square deviation is only 0.12 Å between the Ca atoms 1–427 of AroA. Apparently, formation of the tetrahedral intermediates does not perturb the overall structure of the closed enzymes.

Using the tetrahedral PEP C-2 as center of reference, it is

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* S. Eschenburg and E. Schönbrunn, unpublished data.
as participating in the proton transfer toward and from PEP during catalysis (15, 19). One of the carboxyl oxygen atoms of Glu-341 is in hydrogen-bonding distance to the enol oxygen atom of the PEP moiety, the ε-amino group of Lys-411, and one of the endocyclic nitrogen atoms of His-385. It has been suggested previously that Glu-341 could act as a donor for the initial protonation of the C-3 atom of PEP and could stabilize the positive charge developing at the C-2 of PEP in the addition step of the AroA reaction (19, 33). Moreover, Glu-341 has recently been proposed to also act as the active site base in the elimination step (33), deprotonating the C-3 methyl group of the tetrahedral intermediate in the forward breakdown to the vinyl ether product. A similar dual function has been promoted for Cys-115 in the MurA reaction (15, 34). From the crystal structures presented here, the distances between the C-3 atom of the respective tetrahedral intermediate and the functional groups of the two residues can be measured as 3.4 Å to the closest carboxyl oxygen atom of Glu-341 and 7.0 Å to the sulfhydryl group of Cys-115. Apparently, both residues are too remote from the methyl group at C-3 to act as general acid base catalysts in the MurA reaction.

The binding sites for the first substrates differ substantially in size and structure, accounting for the discrepancy between UNAG and S3P in size, shape, polarity, and charge. In AroA, all polar or charged groups of the S3P moiety are in direct interaction with side chains of the enzyme (Fig. 5, middle). The
The hydrophobic cyclohexene ring of the S3P moiety is held in place through stacked interaction with Tyr-200. Conversely, in MurA, 8 of 11 polar or charged atoms of the UNAG moiety are coordinated through the main chain of the enzyme, either directly or via solvent molecules (Fig. 5, top). Two moderately hydrophobic environments stabilize the orientation of the hexose ring of UNAG pointing toward the 5′ position (Phe-328 and Ile-327, together with the methyl group of Thr-304) and the 1′ position (Trp-95). These hydrophobic sites are absent in AroA. The position of the phenyl group of the Phe-328 of MurA would, in AroA, overlap with the phosphate group of S3P. Instead of Trp-95, AroA contains the strictly conserved Arg-27 (Fig. 5, bottom), which forms a salt bridge to the carboxyl group of S3P and is critical for AroA activity (35).

**Implications for the Catalytic Mechanism**—Without the active site aspartate in D305A-MurA and D313A-AroA, the enolpyruvyl transfer reaction is halted after formation of the tetrahedral adduct of the substrates. Thus, Asp-305/Asp-313 cannot be the base for the initial deprotonation of the target hydroxyl group of UNAG or S3P as suggested previously (11, 19, 36). The nucleophilic attack toward PEP still proceeds if Asp-305/Asp-313 is replaced by Ala, because the tetrahedral intermediates form in the mutant enzymes. Notably, none of the water molecules bound to the active sites of MurA and AroA is located close enough to the enolpyruvyl moiety of the tetrahedral adduct to assist in the proton transfer. In fact, Asp-305/Asp-313 must be responsible for the final proton abstraction from the C-3 atom of the PEP moiety required for the elimination of inorganic phosphate.

When re-introducing the aspartate side chain into positions 305 of MurA or 313 of AroA by homology modeling with the known liganded wild-type structures, the carboxylate group of Asp-305 and Asp-313 would not be closer to the C-3 atom of the tetrahedral intermediate than 3.3 and 3.8 Å, respectively. These rather large distances make it difficult to conceive of a direct attack of the aspartate carboxyl group on the C-H bond.
On the other hand, the carboxylate groups of the modeled aspartate side chains are in good hydrogen-bonding distance of 2.7 Å to the 4'-OH of the hexose and to the 4-OH of the cyclohexene in MurA and AroA, respectively (Fig. 6). Because the 4-OH of the S3P moiety is only 3.1 Å distant from the tetrahedral methyl group (3.4 Å for 4'-OH of the MurA intermediate), the abstraction of a proton from C-3 might proceed intramolecularly if the aspartate carboxylate group abstracts the proton from 4-OH. This hypothesis has not been tested yet, but would be verifiable using 4-deoxy-S3P or 4'-deoxy-UNAG, which, upon reaction with PEP in the respective wild-type enzymes, should yield the tetrahedral intermediate analogs.

From the structure of a fluorinated tetrahedral intermediate analog bound to C115A-MurA, it was concluded that the configuration of the native tetrahedral C-2 of PEP should be 2S in MurA and AroA (15). Our structures corroborate this proposal. Puzzling, however, is the stereochemical course of the addition-elimination reaction. Biochemical analyses of the MurA and AroA reactions have provided strong evidence that addition and elimination proceed with opposite stereochemistry (24). If the addition of the first bound substrate (UNAG or S3P) and a proton to PEP is anti, the elimination of the proton and of inorganic phosphate from the tetrahedral adduct has to be syn, or vice versa. Because the proton addition at C-3 of PEP was believed to be to the si face (15, 37, 38), it was proposed that the enolpyruvyl transfer reactions of MurA and AroA proceed with anti addition and syn elimination, where the proton abstraction from C-3, too, is directed from the si face. The presence of the tetrahedral intermediates in our structures of D305A-MurA and D313A-AroA clearly demonstrates that the side chain responsible for the deprotonation of C-3 is that of the active site aspartate, which points to the re face of PEP (Fig. 7). With the leaving phosphate group on the si face, the elimination step apparently proceeds anti and, consequently, the addition must proceed in syn fashion (Fig. 8). This poses a new view in which the residue possibly acts as the acid in the addition reaction. Apart from Asp-305/Asp-313, only Lys-22 is located on the re side of the PEP moiety (Fig. 7) and is reasonably close to C-2 and C-3. The active site aspartate cannot be the donor for the initial protonation of the C-3 atom of PEP, because the first half of the enolpyruvyl transfer reaction is not hindered in D305A-MurA and D313A-AroA. By the same token, the active site aspartate cannot be the base that deprotonates the 5-hydroxyl and 3'-hydroxyl of S3P and UNAG, respectively, prior to the nucleophilic attack toward PEP (11, 19, 36). Because the

**Fig. 6.** Proposed intramolecular proton transfer for the elimination reaction of enolpyruvyl transfer as exemplified for AroA. In this mechanism, Asp-313 would be the base abstracting a proton from the 4-OH of S3P; the resulting oxyanion then would abstract a proton from the C-3 of the PEP moiety.

**Fig. 7.** Spatial arrangement of active site residues of AroA (top stereo pair) and MurA (bottom stereo pair) with respect to the re face and si face of PEP. Re is above the plane defined by C-1, C-2, C-3, and the enol oxygen atom of PEP; si is below. To visualize the plane, a PEP molecule derived from molecular docking studies (Eschenburg et al.; Ref. 20) is shown in transparent gray in the picture of AroA. The color code is otherwise the same as for Fig. 5.
Implications for Drug Design—Trapping the native intermediates of enzymatic reactions and depicting these in crystal structures is a rare and fortunate event because of the inherent instability of such molecules. Because reaction intermediates are considered more complementary to the active site than the ground state of a reaction, the crystal structures of MurA and AroA, with their genuine tetrahedral intermediate bound to the active site, provide an ideal starting point for the rational design of new potent inhibitors with high selectivity for the target enzyme. The presented structures should be readily exploitable to design inhibitors with high specificity for either MurA or AroA. One challenging goal would be the design of compounds that inhibit both enolpyruvyl transferases likewise. Such inhibitors could become powerful antibiotic drugs, because the simultaneous inhibition of two metabolically unrelated but essential enzymes would be difficult to overcome by bacteria through mere mutagenesis. A prerequisite for the design of such an inhibitor is the proper alignment of the active sites of the two different enolpyruvyl transferases, which differ substantially in amino acid composition and spatial arrangement even of conserved residues. With the chiral center at C-2 of the PEP moiety of the tetrahedral adducts, there is, for the first time, a good reference point for the superposition of both active sites. Aligned through this reference point, the crystal structures of the MurA/THI and AroA/THI complexes show that the cyclohexene and hexose moieties of S3P and UNAG, respectively, are superimposable (Fig. 5, bottom). This suggests that any six-member ring with proper stereochemical features of polar functional groups may serve as the basic core structure of an inhibitor common to MurA and AroA.

Further comparison of the active sites of MurA and AroA using the new reference point explains why MurA is not inhibited by glyphosate. Introducing glyphosate into the active site of MurA, maintaining the same spatial orientation as in AroA (19), reveals clash distances between the phosphonate and carboxylate groups of glyphosate with Arg-120 and Arg-371, respectively, and the N-acetyl moiety of UNAG would interfere sterically with the phosphonate group of glyphosate. In the AroA dead end complex, glyphosate appears to be held in place through hydrogen-bonding interactions of its amide group, which is located midway between the target hydroxyl and the carboxylate group of Glu-341. There is no such residue in MurA that could fulfill this role. Thus, binding of a compound similar to glyphosate to MurA would not only require the absence of the N-acetyl moiety of UNAG but also conformational changes of the Arg-120 and/or Arg-371 side chains as well as an additional residue that properly positions glyphosate similarly to Glu-341 in AroA.

A major obstacle for the design of enolpyruvyl transferase inhibitors is that both reactions follow a stringently ordered mechanism in which the active sites must be created first through binding of UNAG or S3P before PEP can bind. This prerequisite seems to be true not only for PEP binding but also for the binding of the known inhibitors fosfomycin and glyphosate (11, 19). Consequently, inhibitors targeting the active site must either be small enough to bind adjacent to UNAG or S3P, like fosfomycin or glyphosate, or mimic the first substrates’ interaction with the open enzyme state. Compounds that block the domain closure required for catalysis could offer an interesting alternative.

Acknowledgments—We thank Melanie Priestman (University of Kansas) and Andreas Becker (University of Kansas) for helpful discussions.

Note Added in Proof—Two papers regarding the THIs of MurA and AroA with different conclusions on the reaction mechanism of these enzymes appeared recently in the following: Byczynski, B., Mizyed, S., and Berti, P. J. (2003) J. Am. Chem. Soc. 125, 12541–12550 and An, M., Maitra, U., Neidlein, U., and Bartlett, P. (2003) J. Am. Chem. Soc. 125, 12759–12761.

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J. Biol. Chem. 2003, 278:49215-49222.
doi: 10.1074/jbc.M309741200 originally published online September 16, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M309741200

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