Site-Directed Mutagenesis of Conserved Amino Acids in the Alpha Subunit of Toluene Dioxygenase: Potential Mononuclear Non-Heme Iron Coordination Sites

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The terminal oxygenase component of toluene dioxygenase from *Pseudomonas putida* F1 is an iron-sulfur protein (ISP<sub>TOL</sub>) that requires mononuclear iron for enzyme activity. Alignment of all available predicted amino acid sequences for the large (α) subunits of terminal oxygenases showed a conserved cluster of potential mononuclear iron-binding residues. These were between amino acids 210 and 230 in the α subunit (TodC1) of ISP<sub>TOL</sub>. The conserved amino acids, Glu-214, Asp-219, Tyr-221, His-222, and His-228, were each independently replaced with an alanine residue by site-directed mutagenesis. Tyr-266 in TodC1, which has been suggested as an iron ligand, was treated in an identical manner. To assay toluene dioxygenase activity in the presence of TodC1 and its mutant forms, conditions for the reconstitution of wild-type ISP<sub>TOL</sub> activity from TodC1 and purified TodC2 (β subunit) were developed and optimized. A mutation at Glu-214, Asp-219, His-222, or His-228 completely abolished toluene dioxygenase activity. TodC1 with an alanine substitution at either Tyr-221 or Tyr-266 retained partial enzyme activity (42 and 12%, respectively). In experiments with [14C]toluene, the two Tyr→Ala mutations caused a reduction in the amount of cis-[14C]-toluene dihydrodiol formed, whereas a mutation at Glu-214, Asp-219, His-222, or His-228 eliminated cis-toluene dihydrodiol formation. The expression level of all of the mutated TodC1 proteins was equivalent to that of wild-type TodC1 as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot (immunoblot) analyses. These results, in conjunction with the predicted amino acid sequences of 22 oxygenase components, suggest that the conserved motif Glu-X<sub>3</sub>-Asp-X<sub>2</sub>-His-X<sub>4</sub>-His is critical for catalytic function and the glutamate, aspartate, and histidine residues may act as mononuclear iron ligands at the site of oxygen activation.

Toluene dioxygenase (TDO), a multicomponent enzyme system in *Pseudomonas putida* F1, initiates the aerobic catabolism of toluene by forming (+)-cis-(5S,2R)-dihydroxy-3-methyloctahexa-3,5-diene (cis-toluene dihydrodiol) (21, 31, 67). TDO transfers electrons from NADH through Reductase<sub>TOL</sub> (56), and ferredoxin<sub>TOL</sub> (57), to the terminal oxygenase component iron-sulfur protein<sub>TOL</sub> (ISP<sub>TOL</sub>) (55). Reduced ISP<sub>TOL</sub> adds dioxygen to the aromatic nucleus to form cis-toluene dihydrodiol (Fig. 1). ISP<sub>TOL</sub> has an α,β<sub>2</sub> subunit conformation (55). The presence of a Rieske-type [2Fe-2S] center in each α subunit (TodC1) is supported by the presence of a characteristic binding motif (11, 40) in the deduced amino acid sequence of todC1 (70) and recent biochemical analyses of purified TodC1 (30).

Purified ISP<sub>TOL</sub> requires exogenous mononuclear iron (Fe<sup>2+</sup>) for optimum activity (55), and TodC1 is thought to contain the iron-binding site. In related dioxygenase systems, the ISP components of naphthalene and phthalate dioxygenases each contain one atom of iron per αβ and α subunit, respectively (4, 15). Mononuclear nonheme iron centers are believed to be the site of molecular oxygen activation (4, 6, 9, 19, 66), but the mechanism by which iron, dioxygen, and the substrate interact to catalyze monoxygenation or cis-dihydroxylation reactions is not understood. Little structural information is available regarding sites that mediate the binding of mononuclear iron in this type of enzyme.

Amino acids that usually function as iron ligands in proteins are cysteine, histidine, aspartate, glutamate, and tyrosine. For example, histidine and glutamate residues are ligands to the diiron center of methane monoxygenase hydroxylase (5, 48) and to Fe<sup>2+</sup> in the active site in 2,3-dihydroxyphenyl 1,2-dioxygenase (24). Histidine and tyrosine residues are ligands to Fe<sup>2+</sup> in protocatechuate 3,4-dioxygenase (43).

The α subunits of all available sequenced terminal oxygenase components contain conserved histidine, tyrosine, glutamate, and aspartate residues near the middle of their amino acid sequences. The two histidine and tyrosine residues seen at His-222, His-228, and Tyr-221 in this region and Tyr-266 in TodC1 have been suggested as mononuclear iron ligands (13, 38, 40), whereas Nakatsu et al. proposed that a conserved aspartate and histidines (corresponding to Asp-219, His-222, and His-228 in TodC1) may be iron ligands in α subunit alignments that do not include TodC1 (39). In this study, we individually replaced each of the above conserved histidine, tyrosine, glutamate, and aspartate residues in TodC1 with alanine by site-directed mutagenesis and determined the relationship between these mutations and the catalytic function of TDO.

**MATERIALS AND METHODS**

Bacterial strains and growth conditions. *Escherichia coli* JM109(DE3) (Promega Corp., Madison, Wisc.) and BL21(DE3) (54) were used for standard cloning and gene expression. JM109[pDTG613] (70) was used for the purification of TodC2. JM109(pGP1-2), which carries todC1 on pPT7-5 (70) and is designated here JM109(pGP1-2)pDTG612C, and *P. putida* F106 (17), a todC2 mutant strain of strain F1, were used as sources of TodC1, ferredoxin<sub>TOL</sub> (TodB), and reductase<sub>TOL</sub> (TodA) in studies on the reconstitution of active ISP<sub>TOL</sub>. In subsequent experiments, JM109(DE3)pDTG628, constructed as described below, was used as a source of TodB and TodA. Where appropriate for plasmid maintenance, antibiotics were used at the following concentrations: ampicillin, 150 μg/ml; tetracycline, 12.5 μg/ml; kanamycin, 100 μg/ml. *E. coli* cultures were
grown in Luria broth medium (12) or terrific broth medium (33), and strain F106 was grown at 30°C in a mineral salts medium (53) containing 10 mM sodium pyruvate and 0.01% yeast extract, with toluene provided in the vapor phase.

Construction of plasmids. Plasmid pALTER-1 (Promega Corp.) contains a multiple cloning site flanked by the opposing SP6 and T7 RNA polymerase promoters. A 1.6-kb EcoRI-PstI fragment carrying the todC1 gene from pDTG601A (70) was cloned into the EcoRI-PstI sites of the pALTER-1 vector, and this plasmid, designated pDTG632, was used as a template for mutagenesis. A 1.7-kb DdeI-SmaI DNA fragment carrying the todA4 gene was purified from pDTG601 (69). The fragment was treated with S1 nuclease (49) to form blunt ends and ligated to SmaI-digested Kp19 (46), forming pDTG627. The todA4 genes were then excised from pDTG627 by digestion with EcoRI and BamHI and inserted into pT7-7 (2), which had been digested with the same enzymes. The resulting plasmid, pDTG626, contains the todA4 genes under the control of the T7 promoter in pT7-7.

Molecular techniques. Plasmid DNA was isolated as described previously (33) or by using the Qiagen Midi Kit (Qiagen, Inc., Chatsworth, Calif.). For sequencing, DNA was further purified by being passed twice through a Centricon-100 filter unit (Amicon, Inc., Beverly, Mass.). Restriction digestions were performed as described by the enzyme suppliers (New England Biolabs, Inc., Beverly, Mass., and Promega Corp.). E. coli transformations were performed by the method of Hanahan (25).

Site-directed mutagenesis. Mutagenesis of todC1 in pDTG632 was carried out with the Altered Sites II in vitro mutagenesis system in accordance with the manufacturer's (Promega Corp.) instructions. Each mutagenic oligonucleotide was designed with a silent mutation that generated a new restriction site (Table 1) to facilitate the identification of clones carrying the desired mutation. The nucleotide sequence of the entire todC1 gene in each mutant was determined. Sequences and phosphorylated oligonucleotides used for mutagenesis were provided by the University of Iowa DNA Facility.

Expression of cloned genes. E. coli BL21(DE3)(pDTG626), JM109 (DE3)(pDTG632), and recombinant TodC1 and TodC1 mutant derivatives were cultured in Luria broth medium at 30°C. Cultures were induced with 200 μM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h when the turbidity at 600 nm reached 0.7. Cells were harvested by centrifugation and stored at −70°C. Control strain JM109(DE3)(pALTER-1) was subjected to the same procedures as the recombinant organisms.

Preparation of cell extracts. Frozen cells were thawed and suspended in 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 6.8) containing 1 mM dithiothreitol. Phenylmethylsulfonyl fluoride and DTTase I were added to final concentrations of 1 mM and 20 μg/ml, respectively. The cells were broken by two cycles through a chilled French pressure cell. The broken cells were centrifuged at 150,000 × g at 4°C for 1 h to remove cell debris and membranes. The supernatant was pelleted in liquid nitrogen and stored at −70°C.

Protein determinations. Protein concentrations were determined by the method of Bradford (7), with bovine serum albumin as the standard.

Fig. 1. Electron flow in the TDO complex.

Determination of conditions for reconstitution of ISP TOL activity. Determination of conditions for reconstitution of ISP TOL activity. Purification of TodC2. TodC2 was purified by anion-exchange chromatography on a 5-ml column of Mono Q HR 5/5 in 20 mM MES, pH 7.0, containing 1 mM dithiothreitol and 0.2 mM Fe(NH4)2(SO4)2·6H2O. The buffer was used to wash unbound proteins from the column (80 ml at a flow rate of 2 ml/min). The column was then inverted, and the wash procedure was repeated. Proteins were eluted from the column with buffer containing 50% (vol/vol) ethylene glycol and 1.0 M ammonium sulfate. Fractions (0.5 ml) were collected and tested for the presence of TodC2 by Western blotting. Fractions were combined, dialyzed, and concentrated in 50 mM MES buffer, pH 6.8.

Enzyme assays. TDO activity was determined by a modification of the procedure described by Wackett (62). Crude cell extract containing TodC1 from JM109(DE3) carrying pDTG632 or its mutant derivatives, a saturating amount of purified TodC2 (3.3 μg), and cell extract of IPTG-induced BL21(DE3)(pDTG626) (1.3 mg containing TodA and TodB) were added to 50 mM MES buffer, pH 6.8, containing 0.25 mM Fe(NH4)2(SO4)2·6H2O. The mixture was incubated at room temperature for 30 min. Reactions were started by the addition of NADH (0.25 mM final concentration) and 20 μl of [14C]-toluene (19 mM, 1.1 × 106 dpm/μl in methanol). Aliquots (10 μl) were applied to thin-layer chromatography (TLC) squares (1.0 cm2) and air dried to remove all remaining [14C]-toluene, and the amount of nonvolatile cis-toluene dihydrodiol formed was determined in a scintillation counter.

Isolation of anti-TodC2 monoclonal antibody. Puriﬁcation of the anti-TodC2 antibody (35) was separated into its subunits by electrophoresis on a denaturing 15% polyacrylamide gel (32). The region of the gel containing TodC2 and mutant forms of TodC2 in cell extracts as described below. Electrofocus and Western blot analyses. Proteins in cell extracts prepared from E. coli transformants expressing either mutant or wild-type todC1 genes were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12% polyacrylamide gels) (32). Protein detection and Western blotting with antibody 301b were carried out as described previously (35). One hybridoma secreting a monoclonal antibody that gave a positive Western blot with TodC1. This antibody, designated 301b, was purified and used to identify wild-type TodC1 and mutant forms of TodC1 in cell extracts as described below. Detection of reaction products. TLC, followed by autoradiography, was used to detect reaction products. Aliquots (8 μl) from the TDO assays described above were applied to the origin of a TLC plate and developed with chloroform-methanol-water (60:40:5, vol/vol/vol). X-ray films were exposed for 5 days before development.

Computerized sequence analysis. Sequence alignments were done with the Fingerprint program by using a gap weight of 3.5 and a gap length of 0.1 (Wisconsin Sequence Analysis Package; Genetics Computer Group, Madison, Wis.).

RESULTS

Puriﬁcation of TodC2. Studies on the function of specific amino acids in TodC1 required the development of an in vitro

TABLE 1. Oligonucleotides used for site-directed mutagenesis

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Mutagenic oligonucleotide†</th>
<th>Restriction site</th>
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<tbody>
<tr>
<td>Glu-214→Ala</td>
<td>5'-GGG AAT TCG CCG GTC ATG TTG CCA GC-3'</td>
<td>BsuBI</td>
</tr>
<tr>
<td>Asp-252→Glu</td>
<td>5'-TTG ACC CCG TAT GTA CCA TGC CCG GAC-3'</td>
<td>Eco4711</td>
</tr>
<tr>
<td>Tyr-221→Ala</td>
<td>5'-GGC GCC ACA TGG TCG ATG CCA CGA CGA CG-3'</td>
<td>SpI</td>
</tr>
<tr>
<td>His-222→Ala</td>
<td>5'-GCC GCA ACA TGT ACC CGG CGG GGA CG-3'</td>
<td>SacI</td>
</tr>
<tr>
<td>His-228→Ala</td>
<td>5'-GCC GCG ACG ACC TCA CGG CTC TCT GCG ATC CTG-3'</td>
<td>Eco4711</td>
</tr>
<tr>
<td>Tyr-266→Ala</td>
<td>5'-GGG AGT AGC TTC CGG GTC GCC GGC ACC A-3'</td>
<td>BstEII</td>
</tr>
</tbody>
</table>

† The underlined bases represent the restriction sites. The base changes are in boldface.

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Reconstitution of active ISP<sub>TOL</sub> from its TodC1 and TodC2 subunits. Purified TodC2 was preincubated with cell extracts containing TodC1, TodA, and ferrous iron as described in Materials and Methods. Temperature, pH, and preincubation times were optimized. Maximum ISP<sub>TOL</sub> activity was observed after a 30-min incubation period and remained unchanged for up to 8 h. In the following experiments with wild-type TodC1 and mutant forms of TodC1, all dioxygenase components were preincubated in the presence of ferrous iron for 30 min prior to the determination of ISP<sub>TOL</sub> activity. In addition, cell extracts from IPTG-induced cells of JM109 (DE3)(pDTG628) were used as a source of TodA and TodB since they gave twice the ISP<sub>TOL</sub> activity given by extracts from strain F106 (data not shown).

Site-directed mutagenesis of conserved amino acids. The locations of the two proposed iron-binding regions in TodC1 are shown in Fig. 3A. Amino acid sequence alignments of TodC1 and all available related oxygenases were carried out to locate conserved amino acids that might be involved in iron binding. Figure 3B shows the region containing conserved glutamate, aspartate, tyrosine, and histidine residues which are the potential sites for mononuclear iron coordination. Tyr-266, which is not highly conserved throughout the dioxygenases in...
In this alignment, was proposed as an iron ligand on the basis of different alignments of selected sequences (13, 40). To determine whether amino acids Glu-214, Asp-219, Tyr-221, His-222, His-228, and Tyr-266 are essential for catalytic activity, we changed each of them independently to alanine by site-directed mutagenesis. The six individual mutants were isolated, and the position of the alanine substitution was verified by determination of the entire nucleotide sequence of each mutant todC1 gene.

Expression of mutant and wild-type todC1 genes. The wild-type and mutant todC1 genes were each expressed separately in JM109(DE3) under the control of the T7 promoter in the vector pALTER-1. High-level expression of soluble TodC1 proteins from both the wild-type and mutant todC1 genes was achieved. As shown in Fig. 4A, SDS-PAGE analysis of crude cell extracts of the wild type and the mutants revealed equivalent levels of full-length TodC1, corresponding to the calculated 50.9-kDa molecular mass. No band was observed for negative control strain JM109(DE3) (pALTER-1). The Western blot analysis of an exact replica of the SDS-PAGE gel. Monoclonal antibody 301α was used to detect the TodC1 polypeptide.

TDO activities with wild-type and mutant TodC1 proteins. E. coli crude cell extracts containing wild-type and mutant TodC1 were each analyzed for TDO activity in the presence of TodA, TodB, and TodC2 as described in Materials and Methods. As shown in Table 2, the crude wild-type TodC1 protein, when reconstituted with TodC2, gave a TDO specific activity of 469 mU mg⁻¹, which compares favorably with the activity of 2,580 mU mg⁻¹ recently reported for purified ISP₅₉₁ (35). When amino acids Glu-214, Asp-219, His-222, and His-228 were individually mutated to alanine, no activity was detected, while mutations at both Tyr-221 and Tyr-266 resulted in partial TDO activity.

Detection of reaction products. To confirm the results in Table 2, TLC, followed by autoradiography, was used to show that toluene is oxidized to cis-toluene dihydrodiol (Fig. 5). The minor amounts of o-cresol detected are due to nonenzymatic dehydration of the dihydrodiol (21). Replacement of Tyr-221 and Tyr-266 in TodC1 reduced, but did not eliminate, the formation of cis-toluene dihydrodiol, whereas replacement of Glu-214, Asp-219, His-222, and His-228 completely eliminated dihydrodiol formation.

DISCUSSION

The catabolism of aromatic hydrocarbons and related compounds by aerobic bacteria is often initiated by multicompo-

![FIG. 4. (A) SDS-PAGE analysis of wild-type and mutant TodC1 proteins. The gel was stained with Coomassie brilliant blue R250. Lanes: 1, purified ISP₅₉₁; 2 to 9, E. coli cell extracts containing wild-type or mutant TodC1; 2, wild-type TodC1; 3, Glu-214→Ala; 4, Asp-219→Ala; 5, Tyr-221→Ala; 6, His-222→Ala; 7, His-228→Ala; 8, Tyr-266→Ala; 9, cell extract from JM109(DE3) carrying the pALTER-1 vector with no todC1 gene insert. (B) Western blot analysis of an exact replica of the SDS-PAGE gel. Monoclonal antibody 301α was used to detect the TodC1 polypeptide.](image)

![FIG. 5. Autoradiogram of the products formed from [¹⁴C]toluene by TDO with wild-type and mutant TodC1 proteins in the TDO assay system described in Materials and Methods. Lanes: 1, wild-type TodC1 cell extract; 2, Glu-214→Ala; 3, Asp-219→Ala; 4, Tyr-221→Ala; 5, His-222→Ala; 6, His-228→Ala; 7, Tyr-266→Ala; 8, cell extract from JM109(DE3)(pALTER-1). Arrows: o, origin of TLC plate; sf, solvent front; A, o-cresol; B, cis-toluene dihydrodiol.](image)

![TABLE 2. TDO activities obtained with wild-type and mutant TodC1 proteins](table)
nent oxygenases. More than 23 oxygenase systems have been identified by protein purification and/or predicted amino acid sequence analysis. The dioxygenases have been divided into three classes with subgroups based on the number and nature of the electron transport components in each system (3). A comparison of the terminal oxygenase components of dioxygenase and monooxygenase systems reveals one class that consists of dissimilar subunits, α and β, which form an αββ2 or αββ2 conformation, depending on the enzyme. Toluene (55), naphthalene (15), biphenyl (16, 23), benzene (69), benzoxide (66), and toluate (26) dioxygenases are examples of enzymes that belong to this group. In a second class, the terminal oxygenase components consist of identical (αα) subunits, where n = 2, 3, or 4. This group includes phthalate 4,5-dioxygenase (4), 4-methoxybenzoate monooxygenase (6), vanillate demethylase (8), 4-chlorophenylacetate 3,4-dioxygenase (36), 3-chlorobenzozate 3,4-dioxygenase (35), and 4-sulfobenzoate 3,4-dioxygenase (34). Extensive biophysical studies on phthalate 4,5-dioxygenase (19 and references cited therein), 4-methoxybenzoate monooxygenase (64), benzene dioxygenase (20), and benzoxide dioxygenase (66) have shown that each of the α subunits in these enzymes contains a Rieske [2Fe-2S] cluster (47) and mononuclear iron. The latter is believed to be the site of oxygen activation and catalysis.

A comparison of the predicted amino acid sequences for the α subunits in the (αα) and (ααα) oxygenases reveals a great deal of amino acid conservation within each group, while there is little overall conservation between the two groups. The exceptions are at the Rieske center-binding site (11, 40), where the motif Cys-X-His-X16−17-Cys-X2-His is conserved in all of the strains listed in Fig. 3B and in the region we propose to contain the mononuclear-iron-binding site, where the common motif Glu-X3−4-Asp-X2-His-X3−4-His is seen in both classes of terminal oxygenase components (Fig. 3B). To investigate the putative role of these amino acids in TDO activity, we developed an assay that involves the reconstitution of active ISP_TOL from separate sources of its TodC1 and TodC2 subunits. This assay was essential for determination of the activity of mutant forms of TodC1. Similar experiments have been reported for the reconstitution of the terminal oxygenase component of naphthalene dioxygenase (58). However, TodC2 is the first small subunit of an (αβ) oxygenase to be purified and in addition to its importance in the present work, it will provide the basis for future studies on the function of the small subunit in ISP_TOL activity. Harayama et al. (27) have suggested that the isofunctional small subunit in toluate 1,2-dioxygenase plays an important role in the determination of substrate specificity.

Results of site-directed mutagenesis experiments indicate that replacement of the Glu-214, Asp-219, His-222, and His-228 residues of TodC1 with alanine totally abolished TDO activity. However, alanine substitution at either of the two semiconserved tyrosines (Tyr-221→Ala and Tyr-266→Ala) resulted in proteins that retain partial TDO activity. Results of cis-toluene dihydrodiol formation (Fig. 5) were consistent with the results of enzyme assays in that TodC1 proteins with mutations at Glu-214, Asp-219, His-222, and His-228 formed no dihydrodiol, while the protein with mutations at Tyr-221 and Tyr-266 formed reduced amounts of this product. All mutant todC1 genes expressed high levels of the full-length TodC1 protein (Fig. 4), indicating that loss or reduction of TDO activity was not due to truncated, unstable, or reduced levels of TodC1. Taken together, these results demonstrate that Glu-214, Asp-219, His-222, and His-228 are essential for catalytic activity and may act as mononuclear iron ligands. Iron is very loosely bound to ISP_TOL and significant enzyme activity is observed only when ferrous iron is added to the assay compo-

nents (67). Thus, we cannot unequivocally state that these amino acids are involved in iron coordination. However, the role of glutamate, aspartate, and histidine residues in the coordination of mononuclear iron has been well documented for several enzymes, including the catechol ring cleavage enzymes, catechol 2,3-dioxygenase (51), 2,3-dihydroxybiphenyl 1,2-dioxygenase (24, 50), and protocatechuic 3,4-dioxygenase (43). Thus, our results are consistent with the role of these amino acids as mononuclear iron ligands in TodC1. The results are also consistent with the suggestion that the conserved motif Asp-X4-His-X3-His in the deduced amino acid sequences of three (αα) oxygenases may serve as a binding site for mononuclear iron (39). Tyrosines at Tyr-221 and Tyr-266 in TodC1 were proposed as iron ligands (13, 38, 40) on the basis of alignments with fewer sequences than those shown in Fig. 3B. Although Tyr-221 is conserved in all (ααα) oxygenases, it is not present in three of the (ααα) oxygenases (39) (Fig. 3B). In addition, Tyr-266 is only semiconserved in the alignments shown in Fig. 3B. Replacement of either of these two tyrosines with alanine resulted in proteins that retain partial TDO activity and are thus unlikely to interact directly with mononuclear iron. It is possible that the alanine substitution at Tyr-221 may distort the iron-binding site since tyrosine is adjacent to the essential histidine at position 222 in TodC1. Tyr-266 clearly plays an important role in ISP_TOL activity, since its replacement with alanine leads to a dramatic loss in the amount of toluene converted to cis-toluene dihydrodiol. In some sequences, Tyr-266 is replaced with phenylalanine or tryptophan, suggesting that there may be a structural requirement for an aromatic amino acid in this region.

Recent magnetic circular dichroism studies of the dioxygen-activating site of phthalate dioxygenase from P. cepacia (terminal oxygenase has an αα conformation) indicate that there is one six-coordinate Fe3+ site. Substrate binding converts the site to a five-coordinate species, opening up a coordination position for O2 binding (19, 45). However, no data are available that identify the coordinating amino acid residues. Here we present evidence that in the related TDO system, Glu-214, Asp-219, His-222, and His-228 may be at the dioxygen activation site and play a role as mononuclear iron ligands. These amino acids are also contained in the motif Glu-X3−4-Asp-X2-His-X3−4-His, which is conserved in all oxygenase sequences that have been reported to date. The only exception is the presumably allowable substitution of aspartate for glutamate in vanillate demethylase (Fig. 3B). It will be of interest to determine whether the remaining ligands are amino acid residues or water molecules. The latter have been shown to coordinate metals in certain proteins (42, 43, 48). Although the amino acid residues that act as mononuclear iron ligands in dioxygenase α subunits have been proposed for several years on the basis of sequence alignments (13, 38–40), this is the first study that has provided experimental evidence of the importance of these individual residues by replacing each with an alanine. The development of a reconstitution assay for ISP_TOL with purified TodC2 paves the way for further studies of the role of Glu-214, Asp-219, His-222, and His-228 in TDO activity. To establish unambiguously that these amino acids are involved in the coordination of mononuclear iron, the individual TodC1 mutant proteins are being purified and the role of iron in catalysis will be determined by appropriate spectroscopic procedures (19, 45, 51). In addition, changing the above amino acids to residues other than alanine will provide more insight into the oxygen fixation reactions catalyzed by these unique enzymes.
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