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NOTE

Involvement of Electrostatic Interactions between the Components of Toluene Dioxygenase from *Pseudomonas putida* F1

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Abstract A carboxyl group modifier, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was used to study the interactions between three components of toluene dioxygenase (TDO) from *Pseudomonas putida* F1. Ferredoxin$_{TOL}$ activity was increased by the treatment with EDC; however, the activity was rapidly declined in the prolonged incubation. In covalent cross-linking experiments with EDC, Ferredoxin$_{TOL}$ made a one-to-one complex with Reductase$_{TOL}$ or the large subunit of ISP$_{TOL}$. These results provide evidence for the involvement of electrostatic interactions in the TDO electron transfer system.

Key words: *Pseudomonas*, toluene dioxygenase, protein-protein interaction, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, cross-linking

Toluene dioxygenase (TDO) from *P. putida* F1 catalyzes the oxidation of toluene to (+)-*cis*-(1*S*,2*R*)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene 2,3-dihydrodiol) in the presence of dioxygen and NADH [8]. It also catalyzes the oxidation of a variety of aromatic compounds including the structurally unrelated substrate, trichloroethylene [9]. TDO consists of a 46-kDa Reductase$_{TOL}$ which contains FAD [23], a 15.4-kDa Ferredoxin$_{TOL}$ which contains a Rieske-type [2Fe-2S] redox center [24], and a 151-kDa oxygenase (ISP$_{TOL}$) which contains a Rieske-type [2Fe-2S] redox center and mononuclear iron in each large subunit of an $\alpha_2\beta_2$ heterodimer (α = 52.5-kDa, β = 20.8-kDa) [11, 18, 22]. The structural genes for all four proteins of TDO have been cloned and their nucleotide sequences have been determined [30]. Because the reduced Rieske [2Fe-2S] cluster has a spin-coupled [Fe^{2+}-Fe^{3+}] pair [10], Ferredoxin$_{TOL}$ can carry one electron at a time. In addition, Reductase$_{TOL}$ can reduce artificial electron acceptors such as ferricyanide, 2,6-dichlorophenolindophenol and nitrobluetetrazolium [23]. Reduced Reductase$_{TOL}$ can also reduce Ferredoxin$_{TOL}$ which in turn passes electrons to cytochrome *c*. These alternative electron acceptors play an important role in studies on electron transport in TDO and related enzymes. The direction of electron flow in the TDO system is shown in Fig. 1. A family of more than 40 different oxygenases similar to TDO has been identified from various bacteria including *Escherichia coli* in the degradation of natural and xenobiotic aromatic compounds [1, 12]. This indicates that this family of oxygenases plays an important role in the recycling of aromatic compounds in the ecosphere.

In the present study, a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) has been used to investigate the interactions between TDO components. Many studies [4-7, 13, 16, 26, 29] have shown that EDC reacts with carboxyl groups in regions of highly negative-charged environments of proteins followed by either neutralizing the carboxyl groups in the presence (or absence) of an added nucleophile or activating the carboxyl groups to react with an amino group in a charge pair to yield an amide bond, thereby crosslinking components of a protein complex.

Effect of Modification of Carboxyl Groups on the Activities of TDO Components

In order to investigate the effect of carboxyl groups in TDO components on the electron transfer due to protein-protein interactions, EDC was used to neutralize carboxyl groups in the presence of excess glycine ethyl ester. Each TDO component was treated with 5, 10, 20, and 30 mM EDC in the presence of 0.5 M glycine ethyl ester and 20 mM 3-(*N*-morpholino)propanesulfonate (MOPS) buffer, pH 6.8, at 22°C in a total reaction volume of 60 µl. Control experiments were conducted in parallel in the absence of added EDC. Concentrated EDC solutions (50 mM) were prepared in 50 mM MOPS at pH 6.8,

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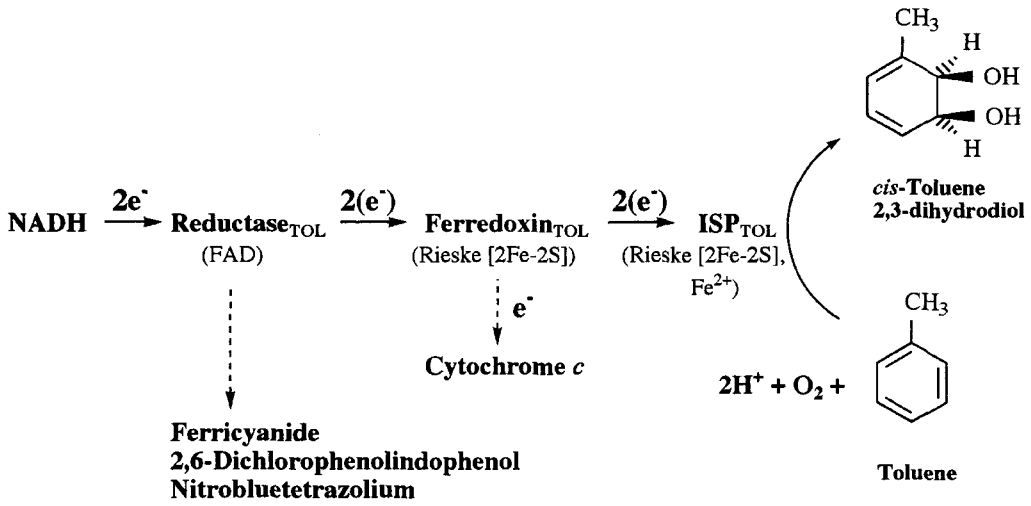

Fig. 1. Proposed electron transport chain and its role in the formation of *cis*-toluene 2,3-dihydrodiol by TDO. e⁻ represents an electron. NADH-chemical reductase and NADH-cytochrome *c* reductase activities are shown by dotted arrows.

immediately before use. Concentrated solutions (2.0 M) of glycine ethyl ester hydrochloride were titrated to pH 6.8 with 10 N NaOH. The concentrations of purified Reductase$_{TOL}$, Ferredoxin$_{TOL}$, and ISP$_{TOL}$ used for the reaction were 4 μM, 22 μM, and 4.5 μM, respectively. Aliquots of the inactivation mixtures were withdrawn at appropriate time points and assayed for the remaining cytochrome *c* reduction and/or TDO activities for a given component. Cytochrome *c* reduction and TDO activities were determined in the presence of 50 mM 2-(*N*-morpholino)ethanesulfonate (MES) buffer, pH 6.8, as described previously [11, 23, 27].

The activity of Ferredoxin$_{TOL}$ which was determined from both cytochrome *c* reduction and toluene dioxygenation assays was increased by the modification (neutralization) of carboxyl groups in earlier incubations with low EDC concentrations, but further reactions of Ferredoxin$_{TOL}$ with EDC resulted in a dramatic decrease in the activity (Fig. 2A). In the cytochrome *c* reduction assay, addition of more than 50 μM cytochrome *c* did not increase the reduction activity in the presence of EDC-modified Ferredoxin$_{TOL}$, indicating that the changes in activity by the modification were due to the changes in the interaction between Reductase$_{TOL}$ and Ferredoxin$_{TOL}$ rather than the changes in the interaction between Ferredoxin$_{TOL}$ and cytochrome *c* in large excess. EDC-treated and -untreated Ferredoxin$_{TOL}$ gave the same visible absorption spectra, suggesting that EDC did not denature Ferredoxin$_{TOL}$ or affect the Rieske-type [2Fe-2S] redox cluster. These results suggest that Ferredoxin$_{TOL}$ has at least two distinct classes of carboxyl groups in both reactivity to EDC and interaction with Reductase$_{TOL}$. In addition, the activity of Ferredoxin$_{TOL}$ was not significantly reduced in the cytochrome *c* reduction assay by the treatment of lysine modifying agents, e.g., 50 mM KOCN and 1 mM 2,4,6-trinitrobenzene-1-sulfonic acid for 3.5 h [15], two successive additions of 60 mM dimethylamine borane/ 80 mM formaldehyde for 4 h [6, 7] (data not shown). These results suggest that the carboxyl groups of Ferredoxin$_{TOL}$ are predominantly involved in interactions with its redox partner (s).

Reductase$_{TOL}$ and ISP$_{TOL}$ were also reacted with EDC to determine the effect of neutralization of carboxyl groups on their activities as described above. The modification of carboxyl groups of Reductase$_{TOL}$ resulted in the gradual increase in activity under the same conditions applied for the Ferredoxin$_{TOL}$ reaction (Fig. 2B). For example, the reaction of Reductase$_{TOL}$ with 30 mM EDC for 90 min yielded a 1.25-fold increase in activity. Furthermore, the ISP$_{TOL}$ activity was dramatically increased by the reaction with EDC. For instance, reactions of ISP$_{TOL}$ with 5 mM EDC for 30 min and with 30 mM EDC for 90 min resulted in 1.5- and 2.0-fold increases in activity, respectively. These results may indicate that some carboxyl groups near the binding sites on Reductase$_{TOL}$ and ISP$_{TOL}$ elicit a charge-charge repulsion to interfere with the right protein-protein interactions with other redox partners. These unusual characteristics of TDO components in activities after being modified by EDC may be required to provide better specificity rather than better efficiency in interactions within the TDO system in the cell where other proteins could have similar interactive properties. In another system, a deletion of a C-terminal portion of adrenodoxin (residues 116-128) resulted in a 1.5-fold increase in the binding affinity to cytochrome P-450$_{SCC}$ with no influence on the interaction with respective reductase [3], indicating that some natural proteins involved in electron transfer are suboptimal in complex formation.

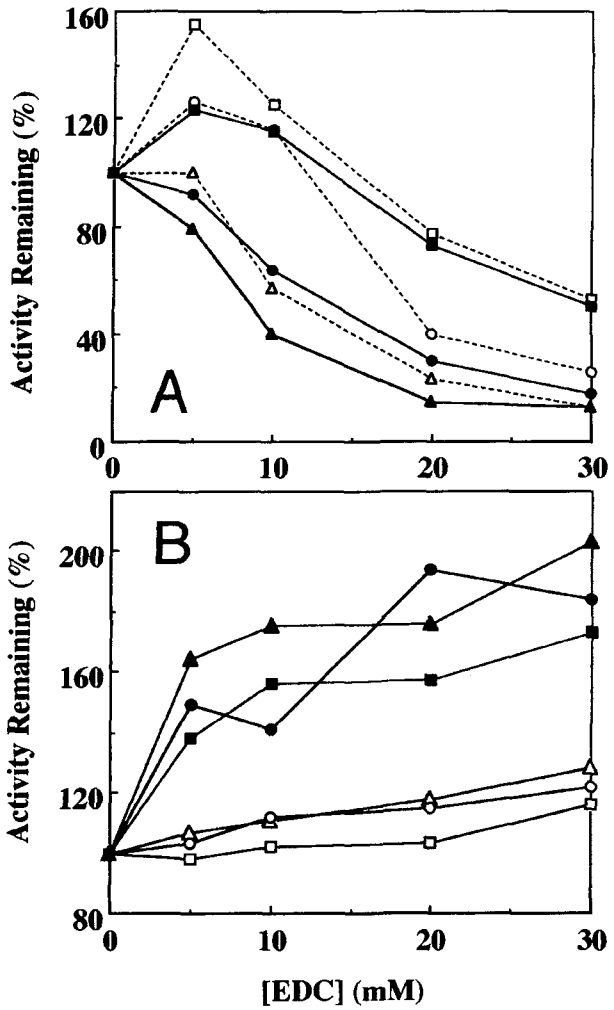

Fig. 2. Changes of $Ferredoxin_{TOL}$, $Reductase_{TOL}$, and ISP_{TOL} activities following treatment with EDC.

The data shown are the means of two different experiments. **A.** $Ferredoxin_{TOL}$ activity was assayed by the cytochrome *c* reduction assay (open symbols) or the TDO assays (closed symbols) after treatment of $Ferredoxin_{TOL}$ for 30 min (□, ■), 60 min (○, ●), or 90 min (△, ▲) with various concentrations of EDC as shown on the abscissa. **B.** $Reductase_{TOL}$ activity was measured by the cytochrome *c* reduction assay and ISP_{TOL} activity by the TDO assay after treatment with various concentrations of EDC as shown on the abscissa: $Reductase_{TOL}$ treated for 30 min (□), 60 min (○), and 90 min (△); ISP_{TOL} treated for 30 min (■), 60 min (●), and 90 min (▲).

Under the same reaction conditions reacted with 5 mM EDC in the absence of added 0.5 M glycine ethyl ester, no increases in activity of TDO components have been observed. But, over a 2 h period, $Ferredoxin_{TOL}$ lost approximately 95% of its initial activity compared to a 10~15% loss for $Reductase_{TOL}$ and ISP_{TOL} (data not shown). These results suggested that, in addition to the neutralization of carboxyl groups of TDO components, intramolecular cross-linking could be another reaction for the inactivation of TDO components by EDC in the absence of added glycine ethyl ester.

Binding Ratios between TDO Components and Identification of Binding Sites

To obtain physical evidence for charge-pairs and binding ratios in the interactions between the TDO components, $Reductase_{TOL}$, $Ferredoxin_{TOL}$, and ISP_{TOL} were incubated with EDC individually or in combination with each other. In this experiment, EDC was used in the absence of an added nucleophile to cross-link a carboxyl group to an amino group in a zero length distance [26]. Thus, the formation of covalent-linked products suggests the involvement of charge-charge (ionic) interactions between the two proteins [5, 13, 29]. Covalent cross-linking between proteins by EDC is based on published methods [5, 29]. TDO components were incubated in the presence of 5 mM EDC in 20 mM MOPS buffer (pH 6.8) in a final volume of 25 μl at 22°C. The quantities of $Reductase_{TOL}$, $Ferredoxin_{TOL}$, and ISP_{TOL} were 1.12, 5.45, and 1.23 nmol, respectively. After a 2 h incubation period, the reactions were quenched by the addition of a sodium acetate solution to a final concentration of 200 mM. One third of the sample was used for separation by denaturing gel electrophoresis on 8~16% linear gradient SDS-polyacrylamide gels (Novex, Encinitas, CA, U.S.A.). Molecular weights of the cross-linked products were determined by comparison of R_f values to those of known standards (Fig. 3).

In the presence of EDC, $Reductase_{TOL}$ (lane 4) and $Ferredoxin_{TOL}$ (lane 5) formed very faint diffusible dimers shown at 90 kDa and 30 kDa positions, respectively. But, they were not considered as active complexes because weak and smeared bands imply nonspecific interactions. However, when the mixture of $Reductase_{TOL}$ and $Ferredoxin_{TOL}$ was treated with EDC (lane 6), a new complex at 56 kDa, corresponding to an intermolecular complex between $Reductase_{TOL}$ and $Ferredoxin_{TOL}$ (RF complex), was observed. When ISP_{TOL} alone was treated with EDC (lane 10), it yielded cross-linked products between the constituent subunits at 104, 80, and 20 kDa positions, corresponding to complexes of the large-large subunit (α_2), the large-small subunit ($\alpha\beta$), and intramolecular cross-linked small subunit (β^*) of ISP_{TOL}, respectively. While no new band was detected in a reaction containing $Reductase_{TOL}$ and ISP_{TOL} (lane 9), a new major band was observed at 62 kDa (lane 8), corresponding to a complex of the large subunit of ISP_{TOL} and $Ferredoxin_{TOL}$ (αF complex). A complex between $Ferredoxin_{TOL}$ and the β subunit of ISP_{TOL} was not observed. In a reaction containing all three TDO components (lane 7), only the RF and αF intermolecular (inter-component) complexes were formed. Furthermore, the same intermolecular cross-linked products were also detected in Western blot experiments with polyclonal antibody raised against purified $Ferredoxin_{TOL}$ (data not shown). Formation of a covalent cross-linked αF

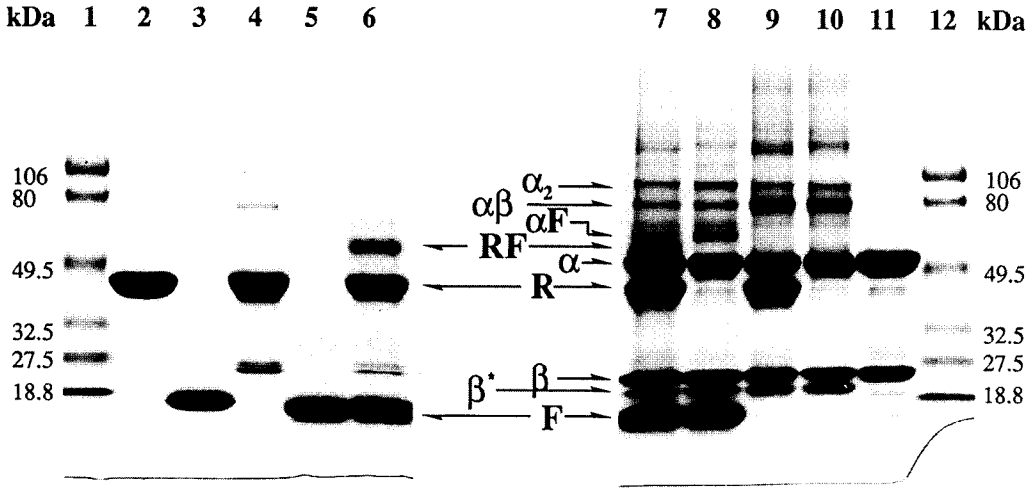

Fig. 3. SDS-polyacrylamide gel (8~16%) electrophoresis of TDO components with and without cross-linking by EDC.

Lanes 1 and 12, prestained molecular weight size markers (Bio-Rad, Richmond, CA); lane 2, $Reductase_{TOL}$ (R); lane 3, $Ferredoxin_{TOL}$ (F); lane 4, $Reductase_{TOL}$ treated with EDC; lane 5, $Ferredoxin_{TOL}$ treated with EDC; lane 6, $Reductase_{TOL}$ and $Ferredoxin_{TOL}$ treated with EDC; lane 7, $Reductase_{TOL}$, $Ferredoxin_{TOL}$, and ISP_{TOL} treated with EDC; lane 8, $Ferredoxin_{TOL}$ and ISP_{TOL} treated with EDC; lane 9, $Reductase_{TOL}$ and ISP_{TOL} treated with EDC; lane 10, ISP_{TOL} treated with EDC; lane 11, ISP_{TOL} (α and β subunits). The designations of the major cross-linked bands are presented: α_2, covalent cross-linkage of two large subunits of ISP_{TOL}; αβ, covalent cross-linkage of the large and small subunits of ISP_{TOL}; αF, covalent cross-linkage of the large subunit of ISP_{TOL} and $Ferredoxin_{TOL}$; RF, covalent cross-linkage of the $Reductase_{TOL}$ and $Ferredoxin_{TOL}$; β*, intramolecular cross-linkage of the small subunits of ISP_{TOL}.

complex, together with the reduced formation of the αβ and β* complexes in the presence of $Ferredoxin_{TOL}$ (Fig. 3, lanes 7 and 8), but not in the presence of $Reductase_{TOL}$ (lane 9), strongly implies that $Ferredoxin_{TOL}$ binds to the large subunit of ISP_{TOL} by salt linkages very near the small subunit of ISP_{TOL}. The formation of an αF complex would facilitate electron transfer from $Ferredoxin_{TOL}$ to ISP_{TOL} since the large subunit of ISP_{TOL} has a [2Fe-2S] cluster [18, 25] which has to associate with the [2Fe-2S] cluster of $Ferredoxin_{TOL}$ for efficient electron transfer.

Because the migration of cross-linked polypeptides cannot identify subunit composition conclusively, the N-terminal amino acid sequences of the products were determined to correlate their molecular weights and binding ratios. A PROTEAN II Slab Cell (Bio-Rad, Richmond, CA, U.S.A.) was used for electrophoretic separation of proteins for N-terminal amino acid sequence determinations. The amino-terminal amino acid sequence of each protein band was determined by Edman degradation using an Applied Biosystems 470A gas phase sequenator (Foster City, CA, U.S.A.).

The 104-kDa product formed from EDC-treated ISP_{TOL} showed only the amino-terminal sequence of the large subunit of ISP_{TOL}. It was assigned as α_2 based on the molecular weight determined by SDS-PAGE. The cross-linked products found at 80, 62, and 56 kDa positions showed mainly 1:1 ratios of α/β, α/$Ferredoxin_{TOL}$, and $Reductase_{TOL}$/$Ferredoxin_{TOL}$, respectively, for each sequencing cycle. However, the 20-kDa product which was formed by EDC-treated ISP_{TOL} could not be sequenced, indicating the amino terminal end of the β subunit is cross-linked to an internal carboxyl group. In this case, it will form a covalent-linked loop resulting in faster mobility than the non-cross-linked β subunit of ISP_{TOL} as shown in Fig. 3. The results obtained by amino terminal sequencing of the complex products confirm the conclusions drawn based on their mobility in SDS-electrophoresis shown in Fig. 3.

Conclusion

Because the loss of electrons in the cell results in the formation of reactive radicals, electron transfer between electron-carrier proteins should be very specific and coupled to a final electron acceptor. For the 'docking' of redox partners, the role of charged groups in other electron transfer proteins has been extensively studied by varying ionic strength [14, 17, 20], through chemical modifications [6, 13], site-directed mutagenesis [2, 20, 21], computer modeling [28], and co-crystallography with redox partners [19]. According to these studies, it has been concluded that electrostatic interactions between redox partners are necessary for both the alignment of redox centers and the formation of a flexible structure necessary for optimal electron transfer. The results showing the changes in activities of TDO components upon EDC treatment (Fig. 2), and the formation of the covalent-cross linked products between TDO components by EDC treatment (Fig. 3), show that electrostatic interactions between TDO components are essential for toluene oxidation.

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