Interaction of the Two Proteins of the Methoxylation System Involved in Cephamycin C Biosynthesis

IMMUNOAFFINITY, PROTEIN CROSS-LINKING, AND FLUORESCENCE SPECTROSCOPY STUDIES*

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Cephamycin C-producing microorganisms contain a two-protein enzyme system that converts cephalosporins to 7-methoxycephalosporins. Interaction between the two component proteins P7 (Mr 27,000) and P8 (Mr 32,000) has been studied by immunoaffinity chromatography using anti-P7 and anti-P8 antibodies, cross-linking with glutaraldehyde, and fluorescence spectroscopy analysis. Co-renaturation of the P7 and P8 polypeptides resulted in the formation of a protein complex with a molecular mass of 59 kDa, which corresponds to a heterodimer of P7 and P8. Glutaraldehyde cross-linking of the polypeptides after assembly of the protein complex showed the presence of a single heterodimer form that reacted with antibodies against P7 and P8. Each separate protein did not associate with itself into multimers. The P7-P8 complex co-purified by immunoaffinity chromatography from extracts of Nocardia lactamurans and Streptomyces clavuligerus, suggesting that both proteins are present as an aggregate in vivo. Fluorescence spectroscopy studies of 5-methylaminonaphthalene-1-sulfonl-P7 in response to increasing concentrations of P8 showed a blue shift in the fluorophore emission, indicating a conformational change of P7 in response to the interaction of P8 with an apparent dissociation constant of 47 μM. NADH showed affinity for the P7 component. The P7-P8 complex interacted strongly with the substrates S-adenosylmethionine and cephalosporin C, differently from that occurring with the separate P7 or P8 components, resulting in a strong blue shift in the fluorescence emission spectra of the complex.

Cephamycin C is a naturally modified cephalosporin antibiotic produced by at least 26 different actinomycetes (particularly species of Streptomyces and Nocardia) (1) that contains a methoxyl group as a substituent at carbon 7 of the cephem nucleus. The presence of a C-7 methoxyl group makes cephamycin resistant to the inactivation by microbial β-lactamases.

The cephamycin C biosynthetic pathway starts with the condensation of three amino acids (L-α-aminoacidic acid, L-cysteine, and L-valine) to form the δ-(L-α-aminoacidylyl)-L-cysteinyl-

d-valine (ACV) tripeptide, the reaction being catalyzed by a large multienzyme peptide synthetase (ACV synthetase) (2). The characteristic cephem nucleus of cephamycin is synthesized from ACV by the consecutive action of (i) isopenicillin N synthase; (ii) isopenicillin N epimerase, (iii) deacetoxycephalosporin C synthase (expandase), and (iv) deacetoxycephalosporin C hydroxylase (for reviews, see Refs. 3–5). After formation of the cephem nucleus, a group of cephem-modifying enzymes (encoded by the "late" genes of the cephamycin pathway) are involved in the C-3' carbamoylation (6) and C-7 methoxylation (7), to form cephamycin C. These enzymatic activities are particularly important because they are responsible for the introduction of chemical substituents into the cephem ring, which modify the pharmacological characteristics of the final antibiotic. All the genes required for cephamycin biosynthesis are located in a single cluster in Nocardia lactamurans (8) and in Streptomyces clavuligerus.2

Synthesis of the methoxyl group is catalyzed, during cephamycin C biosynthesis, by a two-protein (P7 and P8) system, encoded by the genes cmeI and cmeJ of the cephamycin C biosynthetic cluster (7). The methoxylation reaction takes place in two steps. First, the C-7 of the cephem ring is hydroxylated by an α-ketoglutarate- and NADH-dependent hydroxylase, and after this process the hydroxyl group is methylated by a methyltransferase using S-adenosylmethionine as methyl donor (9–11). The hydroxyl group at C-7 derives from molecular oxygen (12). Based on amino acid sequence homologies, one of the components, P7 (encoded by cmeI), appears to have catalytic centers for the two enzymatic activities described above, and the other component, P8 (encoded by cmeJ), may act as a helper protein involved in the physical translocation of the substrate from one catalytic center to another (7).

The characterization of two-component or multienzyme complexes involved in antibiotic biosynthesis opens new perspectives to understand channeling of substrates through the multistep biosynthetic pathways of antibiotics. Knowledge of the molecular mechanism of the methoxylation reaction would facilitate the application of this enzymatic process for the design and synthesis of new β-lactamase-resistant 7-methoxycephalosporins.

In this report, we describe the characterization of the N. lactamurans methoxylation system and provide evidence of the interaction of its protein components by chemical cross-linking and immunoaffinity chromatography; a quantitation of

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1 The abbreviations used are: ACV, α-aminoacidylyl-cysteinyl-valine; CPC, cephalosporin C; FPLC, fast protein liquid chromatography; MOPS, 3-(N-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; dapsyl, 5-methylaminonaphthalene-1-sulfonyl.

2 F. J. Pérez-Llarena, J. F. Martin, and P. Liras, unpublished results.
the interaction has been made by fluorescence spectroscopy studies.

**EXPERIMENTAL PROCEDURES**

**Purification of the Methylation System Components $P_7$ and $P_8$—**To prepare constructions for expression in Escherichia coli, the open reading frame cmc of N. lactamdurans cloned into the plp2291 and the following synthetic oligonucleotides as primers: Oi, 5’-AGATCTGACACACCGGCGCA-3’ and Oii, 3’-CAGCAGCGTGCTCGACATTT-5’, for the cmc gene; and Oiii, 5’-GTCGCCAACCGTGCGACTG-3’ and Oiv, 3’-CGGGAGGTCTCTTCTGAATTT-5’, for the cmc gene.

Polymerase chain reaction conditions were as follows: denaturation 96°C, 30 s; annealing 62°C, 30 s; and extension 76°C, 90 s. The corresponding amplified DNA fragments were purified by agarose electrophoresis, digested with HindIII, and cloned into the restriction sites XmnI and HindIII of pMAL-c2 (an expression vector which allows synthesis in E. coli of foreign peptides by fusing them to a maltose-binding protein) (New England Biolabs) (13). The maltose-binding protein fusion proteins were purified by affinity chromatography on an amylose matrix (BioLabs), and the $P_7$ and $P_8$ peptides were excised from the maltose-binding protein by digestion with factor Xa protease, as described by Maina et al. (14). The $P_7$ and $P_8$ proteins purified from E. coli were used in all experiments on protein interaction studies, except in the co-purification of the $P_7$-$P_8$ complex from extracts of N. lactamdurans or S. clavuligerus (see below).

**Polycyclotin Antibodies against $P_7$ and $P_8$**—The purified $P_7$ and $P_8$ proteins obtained by expression in Streptomyces lividans (15) were used for immunization of New Zealand rabbits, by intradermal injection, using the protocol described by Dunbar and Schwoebel (16). For the primary immunization, 500 µl of protein solution in 50 mM MOPS buffer, pH 7.5 (containing approximately 0.5 mg of the protein of interest) were emulsified with the same volume of complete Freund’s adjuvant. This immunization process was repeated every 2 weeks until a total of 2.5 mg of each protein were injected into the rabbits. For the secondary immunizations, the incomplete Freund’s adjuvant was employed. After the immunization was completed, blood serum was collected by centrifugation, and immunoglobulin G was purified by ammonium sulfate precipitation and FPLC affinity chromatography using a protein A-Sepharose column (Pharmacia Biotech Inc.) as described by Harlow and Lane (17). The antibody titers were assayed in small blood samples by immunoblotting.

**Immunoblotting**—Linkage of the corresponding anti-$P_7$ and anti-$P_8$ antibodies to an activated CNBr-agarose matrix was performed using the commercial Aiminlink Plus kit (Pierce Chemical). Two ml of the resin slurry equilibrated with linkage buffer (100 mM sodium phosphate buffer, pH 7.2, containing 150 mM NaCl) were mixed with the same volume of purified IgG solution at 2.5 mg/ml and incubated overnight at 4°C with gentle agitation. The resin was then washed with 10 ml of linkage buffer, treated with a volume of the same buffer containing 100 mM sodium cyanoborohydride as a reductant, and incubated for 4 h at 4°C with gentle rocking. This reduction process of the free binding sites was repeated twice, and finally, the column was washed with five volumes of 1 M NaCl. The eluants of all the steps of the process were collected to monitor the amount of protein ligated to the resin. After it was prepared, the immunoaffinity resin was loaded into small columns and washed with five volumes of 1 M NaCl to eliminate nonspecific binding to the resin. The columns were stored at 4°C in 50 mM Tris/HCl buffer containing 1 mM sodium azide.

For purification of $P_7$ or $P_8$, cell-free extracts from N. lactamdurans or S. clavuligerus (2-5 mg/ml of protein in 50 mM Tris/HCl, pH 7.6), clarified previously by centrifugation at 15000 × g for 30 min, were applied to the affinity columns equilibrated in the same buffer. After loading the extracts, the columns were washed with 1.5 M NaCl solution, and the proteins retained were eluted with 100 mM glycine/HCl buffer, pH 2.8, collecting 1-ml fractions. The fractions were immediately neutralized by adding 100 µl of 1 M Tris/HCl buffer, pH 8.9, to avoid denaturation of the proteins and analyzed by SDS-PAGE and immunoblotting.

**SDS-PAGE—**SDS-PAGE of proteins was performed as described by Laemmli (18) using a continuous buffer system (50 mM Tris, 150 mM glycine).

**Immunoblotting—**After SDS-PAGE, the proteins were transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore) with a semidy semid electroblotting system (LKB), using a buffer containing 50 mM Tris, 100 mM glycine, and 20% methanol, at a constant current of 0.8 mA/cm². The membranes were then saturated with 1% bovine serum albumin for 1 h at room temperature with gentle rocking and treated with the corresponding antibodies (sera dilution 1/10000) in a 50 mM sodium phosphate buffer, pH 7.6, containing 300 mM NaCl, for 3 h at room temperature with rocking. The membranes were then washed for 15 min with 1 M NaCl and treated with a commercial mouse anti-IgG alkaline phosphatase conjugate (Promega) in the same buffer for 1 h. After another membrane wash with 1 M NaCl for 15 min, the immunoreactive bands were developed with a commercial stabilized substrate solution for alkaline phosphatase (Promega), at room temperature until color appeared.

**Glutaraldehydes Cross-linking**—Cross-linking of the $P_7$ and $P_8$ proteins were performed as described by Eisenstein and Shachman (19), with some modifications. Purified $P_7$ and $P_8$ proteins were concentrated by ultrafiltration in a 50 mM TES buffer, pH 7.5, containing 5 mM urea. Renaturation of the proteins was started by rapid dilution (20-fold) in the same buffer without urea followed by incubation at 30°C in a water bath for 2 h. Periodically, 150-µl aliquots were taken from the samples, and the cross-linking reaction started by adding glutaraldehyde (final concentration 14 mM), and 50 mM borate buffer, pH 8.9. The cross-linking reaction mixture was incubated at room temperature for 20 s, and then the reaction was stopped by adding sodium borohydride (final concentration 10 mM). Cross-linking of the samples was analyzed by SDS-PAGE.

**Protein Labeling with Dansyl Chloride—**$P_7$ and $P_8$ proteins were labeled with dansyl chloride for fluorescence spectroscopy studies. For this, 150 µg of each purified protein in 50 mM MOPS buffer, pH 7.5 (final concentration 100 µg of protein/ml), was treated with 10 µl of a 100 mM dansyl chloride ethanolic solution, and the reaction mixture incubated for 1 h at room temperature. The excess fluorophore was eliminated by serial extractions with ethyl acetate. The dansyl derivatives of both proteins were purified by FPLC gel filtration on a Superose 12 column (Pharmacia), to eliminate the traces of free fluorophore present in the protein solution. The proteins were eluted with a 100 mM MOPS buffer, pH 7.5. Dansylated $P_7$ and $P_8$ proteins showed 7-cephem hydroxylase and methyltransferase activities (up to 90% of the nondonyslated proteins) suggesting that dansylation does not alter the structure of these proteins.

**Fluorescence Spectroscopy Determinations—**Fluorescence determinations of the dansylated $P_7$ and $P_8$ proteins were performed with a SLM Amino 48000 spectrofluorimeter. The protein samples in 50 mM MOPS buffer, pH 7.5, 1 mM dithiothreitol, were gently stirred at 30°C, and excited with a 325-nm light in a quartz cuvette and the emission spectra recorded (scan speed 1 nm/s and bandwidth 5 nm). All studies were carried out with using the same buffer conditions; the fluorescence contribution of the solvent was subtracted.

Kinetic studies for determination of the affinity constants were carried out as described by Otto-Bruc et al. (20). For $K_d$ determination, a constant amount of each dansylated protein was treated with increasing concentrations of ligand, the mixture incubated for 5 min at 30°C to reach equilibrium, and the emission spectrum was monitored. Assuming a simple association model between proteins $P_7$ and $P_8$,

$$P_7 + P_8 \rightarrow P_7P_8$$

(Eq. 1)

the equilibrium constant for the protein-binding process is expressed as $K_d = [P_7P_8]/[P_7][P_8]$. This constant may be expressed with the double-reciprocal plot equation as a function of the modification of the fluorescence intensities of the first protein $P_7$, induced by the presence of increasing amounts of $P_8$:

$$\frac{1}{\Delta F} = \frac{K_d}{\Delta F_{tot}} \left( \frac{1}{[P_7]} + \frac{1}{\Delta F_{tot}} \right)$$

(2)

$\Delta F$ is the increase of $P_7$ fluorescence originated by a specific concentration of $P_8$, and $\Delta F_{tot}$, the maximum increase of $P_7$ fluorescence induced by a saturation concentration of $P_8$ (21) corrected by taking into account the fluorescence modifications induced by the solvent and the added $P_8$ protein alone.

**RESULTS**

**The Cephamycin Methylation System of N. lactamdurans**

Is Composed of Two Subunits $P_7$ and $P_8$, Which Interact In Vitro to Form a Heterodimeric Complex—To elucidate possible interactions between the methylation components, in vivo binding studies were performed using the previously purified...
P7 and P8 proteins. Equimolecular amounts (2.0 mM) of each protein were mixed together in 50 mM MOPS buffer, pH 7.5, containing 5 mM urea as a denaturing agent. The protein renaturation was started by rapid dilution (20-fold) followed by incubation of the solution at 30 °C for 2 h. After this time, the reaction mixture was analyzed by FPLC gel filtration on a Superose 12 column. Results (Fig. 1) showed that co-renaturation of the P7 and P8 polypeptides resulted in the formation of a protein complex (peak C) with a molecular mass of 59 kDa, which corresponds to the approximate sum of the molecular mass of P7 and P8 (27 and 32 kDa, respectively). Addition of detergents, such as SDS, to the renaturation mixture inhibited the protein complex formation, even at low concentrations (0.01%).

The size of the oligomers and the possible formation of higher molecular weight assemblies of the oligomers (unable to enter into the gel filtration matrix) was tested by glutaraldehyde cross-linking of the polypeptides in the assembled protein complex. Equal amounts of each protein were mixed together in 50 mM MOPS buffer, pH 7.5, containing 5M urea as a denaturing agent. The protein renaturation mixture was analyzed by FPLC gel filtration on a Superose 12 column. Results (Fig. 1) showed the presence of a single heterodimer form that reacted with antibodies against each protein. This heterodimer was efficiently formed when the oligomers were co-renatured together, but it was assembled only in small amounts (data not shown) when the experiment was carried out using the native (nondenatured) proteins. These results indicate that the formation of the complex is dependent upon the renaturation degree of the two components, and the complex appears to have a 1:1 stoichiometry, as revealed by the molecular weight of the heterodimer.

Upon prolonged incubation of pure preparations of either P7 or P8 (separately) with the cross-linking agent (under renaturing conditions) no cross-linked dimer of either P7 or P8 was observed, suggesting that each protein does not associate with itself in a multimer form (data not shown).

The Methoxylation System Is a Complex of P7 and P8 Proteins also in Other Cephamycin C Producer Microorganisms—The presence of the P7-P8 complex in cell-free extracts from the cephamycin C-producing actinomycetes (as the result of in vivo cotranslation of the cmc1 and cmcJ genes from a polycistronic mRNA) was difficult to study using conventional chromatography techniques, because of the low abundance of the proteins. Immunochromatography was used to determine if the complex was really formed in vivo. An affinity column containing anti-P7 antibodies linked to the matrix was used to test whether P7 alone, or the P7-P8 complex, was purified from extracts of the cephamycin C producing microorganisms, _N. lactamdurans_ and _S. clavuligerus_. Extracts from 48-h cultures of each microorganism were filtered through the column, and the proteins retained by the antibodies were eluted as described under “Experimental Procedures.” To avoid artifacts, the possible nonspecific binding of the protein P8 to the immunoaffinity column was tested. No retention of the P8 protein by the anti-P7 antibodies in the column was observed.

SDS-PAGE analysis of the fractions eluted from the anti-P7 antibody column (Fig. 3) revealed the presence of two proteins of 27 and 32 kDa, respectively. These polypeptides were characterized as P7 and P8 by immunoblotting with the corresponding antibodies. These results showed that both proteins were...
able to co-purify in the conditions where only purification of P7 was expected, suggesting that P7 and P8 are present as a complex in the extracts. However, the interaction between both subunits appears to be weak, since when stronger washing conditions for the antibody column (2 M NaCl instead 1.5 M NaCl) were used, the P8 protein was unable to co-purify with P7. The same results were obtained when S. clavuligerus cell-free extracts were filtered through the anti-P7 antibody column, indicating the formation of the P7-P8 protein complex in this microorganism.

Fluorescence Spectroscopy of Dansylated P7 in Response to P8 Interaction—Once P7-P8 interaction was established, it was of interest to carry out quantitative studies of the interaction. The fluorescence spectra of a constant amount of dansyl-P7, were recorded in response to different concentrations of the second component P8 (Fig. 4). The maximum emission intensity (at 492 nm) for dansyl-P7 was enhanced in the presence of increasing amounts of P8. In addition, a blue shift in the wavelength for the fluorescence emission maximum was observed, in response to different concentrations of P8 (Fig. 4). This blue shift in the fluorophore emission indicates a conformational change of dansyl-P7, in response to the interaction with P8, which allows the dansyl-group of the fluorophore to be more accessible to the solvent. When a control protein such as bovine serum albumin was added in the same amounts, no fluorescence changes were observed in the dansyl-P7 spectra. A double reciprocal plot of the fluorescence increase of dansyl-P7 in response to increasing concentrations of P8 (Fig. 4B) showed that the dissociation constant of the P7-P8 complex was 47 μM.

Binding of the Reaction Substrates and Cofactors—The same approach was used to determine the binding affinities of the reaction substrates and cofactors for the two components of the methoxylation system, in an attempt to understand the global reaction mechanism. The fluorescence spectra of dansyl derivatives of P7 and P8 were analyzed in the presence of the reaction cofactors (α-ketoglutarate, NADH) and substrates (CPC, S-adenosylmethionine) (7, 10), using each monomer alone or the two-component (heterodimer) system. The dansyl derivatives of the separate P7 monomer, showed very small affinity for the substrates of CPC and S-adenosylmethionine. However, significant changes in the fluorescence spectra of dansyl-P7 were observed in the presence of increasing amounts of NADH (Fig. 5) or α-ketoglutarate (not shown). These changes resulted in a blue shift of its fluorescence emission spectrum, proportional to the NADH or α-ketoglutarate concentration with an estimated K_d value for NADH of 0.293 μM.

The P7-P8 complex was able to interact strongly with the substrates S-adenosylmethionine and CPC, differently from that which occurred with the separate P7 or P8 component. This interaction produced an intense blue shift in the fluorescence emission spectra (Fig. 6). The calculated K_d values were 0.073 μM for S-adenosylmethionine and 0.995 μM for CPC. In summary, the integrity of the protein P7-P8 complex appeared to be a decisive factor for the recognition of the substrate and cofactors by the polypeptidic chains.

DISCUSSION

Interaction between proteins in biosynthetic pathways is a subject of great interest. Metabolic channeling mechanisms in which there is a transfer of reaction products between active centers may result from enzyme-enzyme interaction so that the intermediates are not released into the cytoplasm and cannot be used by enzymes outside the channel (22). Antibiotics and other secondary metabolites are synthesized by multistep path-
ways that are encoded by clustered sets of genes (23). The diversity of amino acid and peptide modifying enzymes involved in the biosynthesis of $\beta$-lactams and other secondary metabolites is extraordinary (24–26).

Introduction of a methoxyl (–OCH$_3$) group converts cephalosporins into the $\beta$-lactamase-resistant cephamycins (4). Previous studies on the characterization of $\text{cmc}_I$ and $\text{cmc}_J$ genes from $N. \text{lactamdurans}$ and their expression in $S. \text{lividans}$ (7) showed that formation of the two enzymatic activities involved in the C-7 methoxylation of the cepham nucleus was dependent upon the co-expression of the two genes in the same vector. Expression of the $\text{cmc}_I$ gene alone resulted in a residual 7-cephem-hydroxylase activity, but it was impossible to detect any 7-hydroxycephem methyltransferase activity. All these data suggested that the methoxylating system consists of two proteins (P$_7$ and P$_8$) and that an interaction occurs between the two components.

Protein affinity chromatography coupled to immunodetection of component polypeptides in the assembled complex and protein cross-linking with glutaraldehyde are well known tools to study protein-protein interactions (27). Using co-renaturation and glutaraldehyde-cross-linking of P$_7$ and P$_8$, we have observed that a heterodimer of these two polypeptides is formed with a 1:1 stoichiometry. Formation of the protein complex was dependent upon renaturing both components together. It is likely that the in vivo assembly of the complex occurs simultaneously with folding of the nascent P$_7$ and P$_8$ proteins, since both polypeptides are co-translated from a single transcript.

The P$_7$-P$_8$ heterodimer co-purifies as a complex as determined by immunoaffinity chromatography. The same results were obtained using extracts of $N. \text{lactamdurans}$ or $S. \text{clavuligerus}$, suggesting that interaction of the two proteins of the methoxylation complex occurs in different cephamycin producers. Methoxylation systems occur in many other actinomycetes (1), and all of them may contain similar two-protein systems, although there is no firm evidence at this time in other actinomycetes to support this hypothesis.

When the association between the monomers is weak, one of the best techniques to study protein-protein interaction is fluorescence spectroscopy, because it is sensitive and nondestructive for proteins (28, 29). Studies involving protein fluorescence may be performed using intrinsic tryptophan fluorescence. However, this method is only suitable for a minor number of proteins which contain few tryptophan residues. In our case, the presence of several tryptophans in the P$_7$ and P$_8$ proteins prevented the use of this approach. Instead proteins P$_7$ and P$_8$ were dansylated with dansyl chloride, and the dansyl group was used as a fluorophore.

Fluorescence spectroscopy studies confirmed that P$_8$ interacts with P$_7$. A blue shift was also observed in the fluorescence spectra of dansyl-P$_7$ in response to increasing concentrations of NADH and $\alpha$-ketoglutarate. The P$_7$ component appears therefore to catalyze electron transfer from $\alpha$-ketoglutarate (oxidized to succinate and CO$_2$) and/or NADH to reduce the molecular oxygen used in the hydroxylation. This result explains the residual 7-cephem-hydroxylase activity observed in the $S. \text{lividans}$ transformants expressing the separate $\text{cmc}_I$ gene (7).
An interesting observation was obtained when the effect of the substrates on the behavior of the P₂-P₈ protein complex was analyzed by fluorescence spectroscopy. The P₂-P₈ complex showed a strong interaction with S-adenosylmethionine and CPC which was not observed with the separate P₂ or P₈ components. These results suggest that interaction of the P₂ and P₈ proteins is required to obtain the proper configuration of the enzyme for efficient recognition of both substrates.

Although there is firm support for the interaction of P₂ and P₈, the exact role of these two proteins in the reactions involved in the introduction of the methoxyl group is still unclear. Efficient hydroxylation at C7 and transfer of the methyl group from S-adenosylmethionine require both P₂ and P₈, although P₂ alone shows weak C7 hydroxylase activity (7). Protein P₂ contains domains that correspond to conserved sequences in catechol-O-methyltransferases by comparison with the crystal structure of cholesterol 7α-monoxygenases and to the active center of O-methyltransferases by comparison with the crystal structure of catechol-O-methyltransferase. Protein P₈ may act as a coupling protein for efficient hydroxylation at C7 in a form similar to that of the two-component system of the Pseudomonas putida p-hydroxyphenylacetate-3-hydroxylase (30). Two- and three-component hydroxylase systems are known to be involved in the oxygenation of sulfobenzoate (31) and dibenzofuran (32), and it is likely that similar systems occur among the widely distributed mono- and dioxygenases existing in nature.

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