The Role of the Conserved Residues His-246, His-199, and Tyr-255 in the Catalysis of Catechol 2,3-Dioxygenase from Pseudomonas stutzeri OX1*§

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Catechol 2,3-dioxygenase (C2,3O) from Pseudomonas stutzeri OX1, which is able to grow on various aromatic substrates as the sole source of carbon and energy, has been expressed in Escherichia coli, purified, characterized, and found to be very similar to other dioxygenases from Pseudomonas species. Interestingly, the activity of the protein shows a rather unusual pH dependence when assayed on catechol. A model of the catalytic mechanism was developed that is able to reproduce the catalytic behavior of the protein as a function of the pH. The model includes multiple equilibria and four productive intermediates with different ionization states of the enzyme-substrate complex. The fitting of the theoretical curve to the experimental data suggests that a tyrosine and two histidine residues are involved in catalysis. Mutants (H246N)-, (H246A)-, (H199N)- and (Y255F)-C2,3O were produced to investigate the role of highly conserved His-199, His-246, and Tyr-255. The strongly reduced activity of the mutants suggests a primary catalytic role for each of these residues. Moreover, mutants at positions 199 and 246 display pH profiles different from that of the wild-type protein, thus indicating that residues His-246 and His-199 play a role in determining the unusual pH dependence of the enzyme. In addition, electron-withdrawing groups on catechol, which increase the acidity of the phenolic hydroxyl group, are able to counterbalance the effect of the mutation H246N in reducing catalytic activity but cause a further reduction of the activity of (H199N)-C2,3O. This finding suggests that His-246 is involved in the initial catechol deprotonation, whereas His-199 promotes the reaction between oxygen and the aromatic ring.

Many soil bacteria can grow on aromatic molecules as the sole source of carbon and energy, revealing an interesting potential for applications in bioremediation strategies (1). Aromatic compounds are first transformed by upper pathways into dihydroxylated intermediates. These intermediates are subsequently metabolized into Krebs cycle intermediates by a limited number of lower pathways (2), which are initiated by ring cleavage reactions that are generally catalyzed by ring cleavage dioxygenases. These enzymes catalyze the addition of two atoms from molecular oxygen into the aromatic ring with a substrate specificity more restricted with respect to that of the upper pathway enzymes. As a consequence, ring cleavage enzymes guide and reduce the metabolic flow of aromatic compounds that can be degraded, thus playing a central role in their catabolism (3).

Ring cleavage dioxygenases acting on catechol and substituted catechols are classified into two groups (4), intradiol dioxygenases and extradiol dioxygenases, with the latter producing 2-hydroxymuconic semialdehydes. The majority of known extradiol dioxygenases belong to a single family of evolutionarily related proteins widespread among prokaryotes (5).

Extradiol dioxygenase activity depends on the presence at the catalytic center of a Fe(II) ion, with the few exceptions of Mn2+-containing dioxygenases (6, 7). The catalytic metal is bound to two histidine residues and one glutamate residue that are well conserved among the members of the family (5). These residues are essential for metal binding and activity (8, 9).

Figs. 1, A and B show a comparison of the active site of 2,3-dihydroxybiphenyl-1,2-dioxygenase (DHBD)† from Pseudomonas sp. KKS102 (8) with that of catechol 2,3-dioxygenase (C2,3O) from Pseudomonas putida mt2 (10). It should be remembered that the structures of the Burkholderia cepacia LB400 DHBD (11), the manganese-dependent 3,4-dihydroxyphenylacetate (homoprotocatechuic) 2,3-dioxygenase (HPCD) from Athrobacter globiformis (12), and the iron-dependent HPCD from Brevibacterium fuscescum have also been determined (12) and found to be very similar to those shown in Fig. 1, A and B.

Multiple alignments show that three other residues also present at the active site, His-246, His-199, and Tyr-255 (P. putida mt2 C2,3O numbering; Fig. 1, A and B), are well conserved (5, 13). Thus, it might be inferred that these residues play a role in substrate binding and/or catalysis. DHBD from Pseudomonas sp. KKS102 (8) and B. cepacia LB400 (11) and HPCD from A. globiformis and B. fuscescum (12) have been crystallized with substrates bound in the active site (Protein Data Bank codes 1KW9, 1KMY, 1KND, 1KNF, 1F1V, and 1QOC). It

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§ The on-line version of this article (available at http://www.jbc.org) contains supplemental material (including Table S1) that elaborates on the reaction scheme in Fig. 3.

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1 The abbreviations used are: DHBD, 2,3-dihydroxybiphenyl-1,2-dioxygenase; C2,3O, catechol 2,3-dioxygenase; CHES, 2-(cyclohexylamino)ethanesulfonic acid; MOPS, 3-(N-morpholino)ethanesulfonic acid; HPCD, 3,4-dihydroxyphenylacetate (homoprotocatechuic) 2,3-dioxygenase; MALDI/MS, matrix assisted laser desorption ionization mass spectrometry; MES, 3-(N-morpholino)propanesulfonic acid; wt, wild-type.
should be noted that in all of the structures the oxygen atom of the substrate that is on the same side of Tyr-249 (Tyr-257 in HPCD and Tyr-255 in C2,3O) is closer to the iron ion than is the other catechol oxygen atom (Fig. 1A), suggesting that only this hydroxyl group is in the anionic form (8, 11). This hypothesis is confirmed by Raman and electronic absorption studies (11). Moreover, extended x-ray absorption fine structure studies indicate that P. putida C2,3O also binds catechol as a monoanion (14). It should also be noted that Tyr-249 has been proposed to be H-bonded to the anionic oxygen of the catechol ring, acting as H donor, whereas His-194 (His-199 in C2,3O from P. putida mt2) is H-bonded to the non-dissociated hydroxyl group, acting as H-bond acceptor (8). Finally, studies on synthetic catalysts that mimic extradiol dioxygenases (15) and studies on the manganese-dependent HPCD (16) indicate that the first deprotonation of catechols is the first crucial step of extradiol cleavage reactions. According to these observations, a mechanism was proposed (8, 10, 11, 17) (Fig. 1C) in which conserved His-246 and Tyr-255, or their homologous residues, act in a concerted way to abstract the first proton from catechol,
whereas His-199, at a later stage, acts as a basic catalyst by accepting the second proton from the substrate. Finally, the position of $O_2$ attack and the mechanism of its insertion in the aromatic ring have been addressed by density functional theory studies (18) and by studies with model compounds and analogues of the hypothetical reaction intermediates (19–21). However, despite the large number of studies, direct evidence of the roles of His-199, His-246, and Tyr-255 in catalysis is still lacking, with the exception of data available on the involvement of His-200 of the iron-dependent HPCD from $B$. fuscum (22).

In this paper we report the overexpression in Escherichia coli and the purification and characterization of the catechol 2,3-dioxygenase from Pseudomonas stutzeri OX1 and four active site mutants designed for obtaining information on ionizable groups involved in catalysis. The catalytic parameters of C2,3O and the mutants were studied on catechol as a function of pH groups involved in catalysis. The catalytic parameters of C2,3O dioxygenase from (22).

In the absence of catalytic groups, the difference between the spectrophotometric titration curves of the product and the substrate. Spectrophotometric titration curves were measured spectrophotometrically as described (28). The theoretical titration curves were fitted to experimental data by the program GraphPad Prism that was also used to calculate the $K_a$ values. Titration was followed at 238 nm for the determination of 3-formylcatechol and 450 nm (pH 7.5–10).

When catechol was used as substrate, the reaction was followed at 375 nm (pH 7.5–10). Tris/His buffers were not used for the determination of formylcatechol titration curves. All of the buffers were freshly prepared, and the pH of the buffer was measured at the final concentration of use. Determination of $M_v$ Value—The $M_v$ value of the native wild-type enzyme was determined by gel filtration on a Superose 12 PC 32/300 (3.2 × 300 mm) column equilibrated in 50 mM Tris/HCl (pH 7.5) containing 0.2 M NaCl, 10% ethanol (v/v), and 5% glycerol (v/v) using a SMART system (Amersham Biosciences). The molecular mass markers used as standards were $\beta$-amylase (M, 200,000 Da), yeast alcohol dehydrogenase ($M_v$, 150,000 Da), and aspartate aminotransferase ($M_v$, 90,000 Da).

Determination of Titrination Curves and $pK_v$ Values—Titration curves were determined spectrophotometrically as described (28). The theoretical titration curves were fitted to experimental data by the program GraphPad Prism that was also used to calculate the $pK_v$ values. Titration was followed at 238 nm for the determination of 3-formylcatechol $pK_v$, 347 nm for the determination of 4-formylcatechol $pK_v$, and 430 nm for the determination of 4-nitrocatechol $pK_v$.

The buffers (50 mM each) used were sodium acetate (pH 4–5.75), MES/NaOH (pH 5.75–6.5), MOPS/NaOH (pH 6.5–7.8), sodium phosphate (pH 6.5–8.2), Tris/Cl (pH 7.1–8.8), and CHES/NaOH (pH 8.6–10). Tris/His buffers were not used for the determination of formylcatechol titration curves. All of the buffers were freshly prepared, and the pH of the buffer was measured at the final concentration of use. Determination of $h_{cat}$ Values as a Function of pH—The variations of $k_{cat}$ values as a function of pH were determined spectrophotometrically. When catechol was used as substrate, the reaction was followed at 375 nm (pH 7.5–10). When 3-formylcatechol was used, the reaction was followed at 400 nm (pH 7.5–10). When 3-formylcatechol was used, the reaction was followed at 300 nm (pH 5–6.8), 425 nm (pH 6.8–7.5), and 450 nm (pH 7.5–10). $\Delta v$ values as a function of pH were calculated from the differences between the spectrophotometric titration curves of the product and the substrate. Spectrophotometric titration curves were described as determined above under "Determination of Titrination Curves and $pK_v$ Values." All $h_{cat}$ values were determined at saturating concentrations of the substrate. Saturation was verified at each pH value by measuring the rate of the reaction at three increasing substrate concentrations.

The buffers (50 mM each) used were sodium acetate (pH 4–5.75), MES/NaOH (pH 5.75–6.5), MOPS/NaOH (pH 6.5–7.8), sodium phosphate (pH 6.5–8.2), Tris/Cl (pH 7.1–8.8), and CHES/NaOH (pH 8.6–10). Tris/His buffers were not used for the determination of $h_{cat}$ values.
Protein concentration was determined colorimetrically with the Bradford reagent (31) using bovine serum albumin as a standard. Total protein concentration was measured at their final concentration.

The molecular mass of denatured C2,3O was determined by high pressure liquid chromatography-purified protein were digested with other extradiol dioxygenases (Table I) indicate that C2,3O belongs to the major family of extradiol ring cleavage dioxygenases that have the substrate binding domain mt2; moreover, the Ferene S assay revealed that recombinant C2,3O is expressed in E. coli with the initial Met residue.

The molecular mass was found to be 117,000 Da. This value is consistent with the hypothesis that C2,3O is a homotetramer, as expected by its homology with other catechol dioxygenases (10).

Sensitivity to oxidation of the wild-type enzyme was verified by incubation in 0.04% H2O2 for 30 min. This treatment resulted in the loss of ~98% of the activity. Incubation of the H2O2-inactivated enzyme in Fe(NH4)2(SO4)2 solutions resulted in the recovery of ~35% of the activity. When Mn2+, Co2+, and Fe3+ salts were used instead, no recovery of the activity was ever detected, thus indicating the specificity of the enzyme for Fe2+. The ratio of moles of iron to moles of a monomer of C2,3O was found to be 1.1 ± 0.02. Moreover, the Ferene S assay carried out with and without the addition of ascorbic acid yielded the same value, thus indicating that iron is present as Fe3+.

The rate of ring fission of wt-C2,3O was determined on catechol and several substituted catechols (Table II). Catechol is the best substrate, followed by 4-methylcatechol and 3-methoxycatechol. Larger groups at positions 3 or 4 cause further reduction of kcat values.

When 4-methylcatechol, 4-ethylcatechol, 3-methoxycatechol, 4-formylcatechol, and 4-nitrocatechol were used as substrates, a rapid inactivation of C2,3O was observed. In fact, the initial reaction rate rapidly decreased to zero. When fresh enzyme was added, the reaction started again, and the total amount of substrate converted was found to be proportional to the amount of the enzyme added. This behavior has already been observed for other C2,3Os and has been attributed to inactivation mediated by the substrate (35–37). The ability of the substrate to

| Protein* | Organism | Substrate | Identity | Accession no.*b |
|----------|----------|-----------|----------|-----------------|
| XyIE     | P. putida mt-2 | Catechol | 95 | P06622 |
| DMPB     | Pseudomonas sp. CF600 | Catechol | 85 | P17262 |
| TODE     | P. putida F1 | 3-Methylcatechol | 27 | P13453 |
| DHBD     | Burkholderia sp. LB400 | 2,3-Dihydroxybiphenyl | 31 | P42228 |
| DHBD     | Pseudomonas sp. RKS102 | 2,3-Dihydroxybiphenyl | 30 | P17297 |
| NAHC     | P. putida G7 | 1,2-Dihydroxynaphthalene | 21 | P11861 |

* XyIE and DMPB, catechol 2,3-dioxygenases; TODE, 3-methylcatechol dioxygenase; NAHC, 1,2-dihydroxynaphthalene dioxygenase.

** Swiss-Prot accession numbers.
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Table III
Kinetic parameters at pH 7.5 of wild-type and mutant C2,3O

| Substrate (pK_a) | Wt-C2,3O | 3-Formylcatechol (7.7) | 4-Formylcatechol (7.2) | 4-Nitrocatechol (6.7) |
|------------------|----------|------------------------|------------------------|-----------------------|
|                   | k_cat (s^-1) |                      |                       |                       |
|                   | K_m (M)    | k_cat/K_m(s^-1) (M^-1) | k_cat/K_m(s^-1) (M^-1) | k_cat/K_m(s^-1) (M^-1) |
| Catechol (9.45)^a | 200 ± 15 (100) | 47 ± 2 (23)           | 16 ± 1 (8)            | 1.4 ± 0.2 (0.7)       |
|                   | 1.5 ± 0.27 | 32 ± 2                 | 168 ± 17              | 35 ± 8                |
|                   | 120        | 1.5                    | 0.985                 | 0.04                  |
| (Y255F)-C2,3O     | 8.8 ± 0.3 (100) | 2.6 ± 0.1 (29)       | 0.45 ± 0.05 (5)       | -                     |
| (H246N)-C2,3O     | 5.0 ± 0.4 | 51.6 ± 5               | 248 ± 20              |                       |
|                   | 1.76       | 0.05                   | 1.8 ± 10^-3           |                       |
| (H198N)-C2,3O     | 0.46 ± 0.05 (100) | 0.03 ± 0.001 (6.5)   | 0.0047 ± 0.0004 (1)   |                       |
|                   | 434 ± 40 | 1170 ± 100             | 600 ± 80              |                       |
|                   | 1.1 ± 10^-3 | 2.6 ± 10^-5           | 7.8 ± 10^-6           |                       |
| (H246N)-C2,3O     | 16 ± 0.1 (100) | 6.9 ± 0.5 (430)      | 0.43 ± 0.03 (27)      |                       |
|                   | 714 ± 70 | 440 ± 64               | 970 ± 98              |                       |
|                   | 2.2 ± 10^-3 | 0.016                 | 4.4 ± 10^-4           |                       |
| (H246N)-C2,3O     | 0.45 ± 0.05 | 5000                 | 9 × 10^-5             |                       |

^a Value from Ref. 11.
^b Errors <0.1 pH unit.
^c Values in parentheses are percentages of the k_cat values of each protein on catechol.

induce inactivation of the enzyme was measured by the ratio between the amount of substrate converted before complete inactivation and the number of micromoles of the enzyme. These ratios, called “partition ratios” (26), are reported in Table II. Note that 4-methylcatechol is a better substrate than 3-methyl and 3-formylcatechol, but it is also more inactivating. It should also be emphasized that all of substituents tested caused an increase in the ability of the substrate to inactivate the enzyme. Moreover, larger groups and groups at position 4 had a more pronounced effect.

Kinetic Parameters of wt-C2,3O—To study the influence of substrate ionization on catalysis and the influence of substituents on the substrate, kinetic parameters of C2,3O were determined both on catechol and on substituted catechols bearing different electronegative groups that increase the acidity of the aromatic hydroxyl groups. pK_a values of substituted catechols were determined spectrophotometrically as described under “Material and Methods” and are reported in Table III.

Table III shows the kinetic parameters determined for wt-C2,3O on catechol and acidic catechols at pH 7.5. It is notable that the k_cat of wt-C2,3O decreases as substrate acidity increases, i.e. as the electron-withdrawing effect of the substituent group increases. In fact the k_cat values for 3-formyl, 4-formyl, and 4-nitrocatechol are 23, 8, and 0.7% of the k_cat value on catechol.

To gain information on the ionizable groups involved in catalysis, k_cat values on catechol and formylcatechols were also determined at pH values between 5 and 10. Fig. 2A shows the apparent k_cat value of wt-C2,3O on catechol as a function of pH. The curve is markedly asymmetric. In fact, it shows a maximum at pH 6, whereas it decreases at lower and higher pH values with very different degrees of steepness. Activity drops quickly at pH values <6, and at pH 5 activity is already near zero. On the other hand, at pH values >6, activity decreases very slowly and a shoulder is present between pH 7.5 and 8.5. To exclude an effect of buffer ions between pH 6.5 and 9, activity was measured using at least two different buffer systems for each pH, i.e. MOPS, sodium acetate, Tris-HCl, or CHES/NaOH. Identical k_cat values were determined (data not shown), thus excluding any buffer effect.

Fig. 2, B and C show the variation of k_cat values on 3- and 4-formylcatechol as a function of pH. The two curves are very similar and bell-shaped, in contrast with the curve obtained using catechol as substrate. For both formylcatechols, k_cat values show a large maximum between pH 7 and 8 that drops at pH values >8, whereas they slowly decrease at pH values <7. This decrease is slower for 4-formylcatechol than for 3-formylcatechol.

To obtain an estimate of the pK_a values of the residues responsible for the shape of the curves shown in Fig. 2, A–C, we developed a model for the reaction of C2,3O with its substrates and derived an approximate mathematical solution that was used to fit the experimental data. The model, shown in Fig. 3, hypothesizes the presence of two different enzyme active forms, EH_H and EH, which depend on the ionization state of three ionizable groups on the enzyme. Each of these two species can productively bind two forms of the substrate, SH and S~ with different affinities (K_{HiSH} and K_{HiS~}). The model also assumes that productive reactions have different k_cat values and that EH_HSH and EH_SH~ species do not directly convert one into the other (Fig. 3).

The derivation of the equation and its fitting to experimental data, described in detail in the supplemental material available in the on-line version of this article, allowed us to obtain three sets of values for the three pK_a values of the groups that influence catalysis. The pK_a values of the two most acidic residues are 5.5 ± 0.1 and 6.6 ± 0.15. These values suggest the involvement in catalysis of two histidine residues likely positioned in different environments. The analysis of the structure of P. putida mt2 C2,3O showed that, excluding the His-153 and the His-214 that bind the iron ion, only two other histidines are placed in the proximity of the active site cavity, His-199 and His-246 (Fig. 1). Thus, these residues can be tentatively identified as the residues with pK_a values of 5.5 and 6.6.

The third group is a weaker acid, the pK_a value of which was determined with lower accuracy and falls in the range between pH 8.9 and 9.9. In principle, this pK_a value could be assigned to a tyrosine, a lysine, or a cysteine residue. Residue Tyr-255, at <4 Å from the iron ion, is a likely candidate.

The fitting procedure also allowed us to obtain information on the k_cat values of the different active forms of the enzyme. When catechol is used as substrate, reaction 2, between EH_H...
and S⁻, gives a negligible contribution because the EH₂ form is present only at pH values in which catechol is completely in its neutral form (Figs. 2A and 3). Therefore the shape of the activity curve on catechol is determined only by the values of $k_{\text{cat},1}$, $k_{\text{cat},4}$, and $k_{\text{cat},4}$. The shoulder at the alkaline pH values depends only on the EH form (Fig. 2A, dotted lines, and Fig. 3). It should be noted that $k_{\text{cat},3}$ and $k_{\text{cat},4}$ values, i.e. the catalytic constants of EH species, are ~2.6–3 times lower than that of

$k_{\text{cat},1}$ (supplemental Table S1, available in the on-line version of this article), indicating that the EH₂ form is more active than the EH form. A different picture emerges from the fitting of the model to experimental data collected using formylcatechols as substrates. In this case, the EH form has $k_{\text{cat}}$ values higher than those of the EH₂ form (Table S1 and the supplemental materials (on-line version of this article), indicating that the EH form is more active on these substrates than the EH₂ form.

**Kinetic Parameters of Mutated C2,3O**—To investigate the role of the conserved residues His-199, His-246, and Tyr-255 in the catalytic process of *P. stutzeri* C2,3O, four mutants were produced by site-directed mutagenesis as described under “Materials and Methods,” namely (H199N)⁻, (H246N)⁻, (H246A)⁻, and (Y255F)⁻ C2,3O. These mutants were expressed, purified, and characterized. All mutated proteins showed an Fe²⁺ content similar to that of wt-C2,3O within the experimental error.

Table III shows the kinetic parameters determined at pH 7.5 using catechol or formylcatechols as substrates. Mutation H199N leads to a 400-fold decrease of the $k_{\text{cat}}$ value on catechol, whereas the $K_m$ value is increased 290 times. Moreover, the inverse correlation between $k_{\text{cat}}$ values and the substrate acidity already observed for the wild-type enzyme is much more evident in the case of the mutant (H199N)⁻ C2,3O. In fact, the $k_{\text{cat}}$ values for 3-formyl and 4-formylcatechol are 6.5 and 1% of those measured for catechol.

On the other hand, the mutation H246N causes a slightly less pronounced decrease of the $k_{\text{cat}}$ value for catechol, which is 125-fold lower than that of wt-C2,3O, but a larger increase of the $K_m$ value, which is 480-fold higher than that of wt-C2,3O. It is noteworthy that the relationship between the $k_{\text{cat}}$ and the substrate acidity of (H246N)⁻ C2,3O is considerably different from that observed in the case of wt- and (H199N)⁻ C2,3O (Table III). In fact, the $k_{\text{cat}}$ value of (H246N)⁻ C2,3O for 3-formylcatechol is ~4-fold higher than that for catechol, whereas wt- and (H199N)⁻ C2,3O show $k_{\text{cat}}$ values for catechol that are 5- to 10-fold higher than those for acidic catechols. Moreover, the $k_{\text{cat}}$ value of (H246N)⁻ C2,3O for 4-formylcatechol is only 3.7-fold lower than that for catechol, in contrast with the 12.5- and 100-times reduction measured in the case of the wild-type protein and the (H199N)⁻ C2,3O mutant, respectively.

As for mutation H246A, it has a dramatic effect on catalysis; the $K_m$ value for catechol increases >3000-fold, and the $k_{\text{cat}}$ value decreases ~450-fold. The mutation Y255F causes a limited effect on the $K_m$ value, which increases only ~3-fold, and a larger effect on the $k_{\text{cat}}$ value, which decreases 25-fold. The relationship between the $k_{\text{cat}}$ values and substrate acidity is very similar to that of wt-C2,3O (Table III).

The dependence of the $k_{\text{cat}}$ values of mutated proteins on pH was studied using catechol as the substrate. The mutants (H246N)⁻ and (H246A)⁻ C2,3O show pH profiles that are different from that of the wild-type enzyme and different from each other (Fig. 2D). Mutant (H246N)⁻ C2,3O shows a maximum at a pH near 8 with a shoulder between pH 8 and 9. Moreover, the maximum at pH 6 is absent. Mutant (H246A)⁻ C2,3O shows a simpler profile with only a wide but very low maximum at pH 8.5–9.

The $k_{\text{cat}}$ values of (H199N)⁻ C2,3O are constant in the entire pH range from 5 to 9 and slowly decrease at higher pH values (Fig. 2D). The $k_{\text{cat}}$ values of this mutant are intermediate between those of (H246N)⁻ C2,3O and (H246A)⁻ C2,3O at all pH values. Finally, (Y255F)⁻ C2,3O shows a pH profile very similar to that of wt-C2,3O but proportionally lower at all pH values (Fig. 2D).

**DISCUSSION**

Catechol 2,3-dioxygenases are key enzymes in the catabolism of aromatics, limiting the number of molecules that can be
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Fig. 3. Scheme of the reactions catalyzed by C2,3O as a function of pH (vertical axis on the left). The bar on the right shows the percentage of catechol in the anionic form (S') as a function of pH. See the supplemental material in the on-line version of this article for details.

The C2,3O from P. stutzeri that we purified is very similar to other well characterized C2,3Os (see Table I), particularly to that from P. putida MT2, with ~95% sequence identity. The differences are mainly located in the N-terminal domain, whereas in the C-terminal catalytic domain of the two proteins only one conservative mutation is present, 14 Å away from the catalytic iron. Thus, P. stutzeri C2,3O also retains the conserved active site residues His-246, His-199, and Tyr-255 for which a role in the catalytic mechanism of dioxygenases has been hypothesized (8, 10, 11). However, it should be emphasized that no direct experimental evidence is available on the enzyme-ionizable groups should have \( pK_a \) values of -5.5, 6.6, and 9–10 (see the legend of Fig. 2), respectively. It should be noted that the two more acidic \( pK_a \) values (5.5 and 6.6) agree with the involvement of two histidine residues in catalysis. Moreover, the presence of a maximum of activity at pH 6 would indicate that the enzyme species present at this pH is the most active one. This form in our model is EH2, which should bear a protonated and a deprotonated residue. These two residues were tentatively identified with the conserved histidine residues 199 and 246, based on the following considerations. The analysis of the structures of C2,3O (Protein Data Bank code 1MPY) and DHBD (Protein Data Bank codes 1KWD, 1KMY, 1KND, and 1KNF) from P. putida shows that the conserved His-199 and His-246 are located in two rather different environments (Fig. 1, A and B). As for His-199, its N2 atom points toward the substrate binding pocket (in DHBD structures it is H-bonded to the OH group of the substrate), whereas its N31 atom points toward a narrow solvent-filled channel (Fig. 1, A and B). As for His-246, it cannot form H-bonds with the substrate, but the possibility exists that it may be H-bonded to Glu-265 through its N1 atom and/or to the backbone CO of Ile-291 through its N31 atom. It can be hypothesized that when His-246 is protonated, both H-bonds are formed (as shown in Fig. 1, A and B), whereas only one H-bond will be formed in its deprotonated form. Thus, based on the close proximity of His-246 to Glu-265 and on its potential to form two hydrogen bonds in its protonated form, it can be hypothesized that His-246 is the protonated histidine in the EH2 species of...
our model. On the other hand, we suggest that His-199 is in its deprotonated form in the EH$_2$ species as required by the mechanism shown in Fig. 1C.

As for the third group with a p$K_a$ value in the range 9–10, Tyr-255, located at <4 Å from the iron ion, is a likely candidate. It should be emphasized that its proximity to the iron ion could explain a p$K_a$ value <10.

To test the hypothesis that the three ionizable groups of our model are the residues His-199, His-246, and Tyr-255, we prepared and characterized mutants (H199N)-, (H246N)-, (H246A)-, and (Y255F)-C2,3O. The kinetic parameters determined at pH 7.5 (Table III) indicate that the mutation H199N influences both $k_{cat}$ and $K_m$ values. It should be noted that the $k_{cat}$ value decreases 430 times, thus indicating that His-199 is very important for catalysis, in line with the result already obtained on HPCD from $B$. fuscaum (22). Moreover, the $K_m$ value increases from 1.5 to ~400 μM. Assuming that $K_m$ values are a measure of substrate affinity, this finding indicates that His-199 is also important in substrate binding, likely because of the formation of an H-bond with the substrate and/or by contacting the catechol ring (Fig. 1A). Moreover, its $k_{cat}$ versus pH profile is flat in the entire pH range tested, and the $k_{cat}$ values are very low at all pH values with respect to those of the wild-type enzyme, as expected for a residue that is involved in catalysis at all pH values. Thus, we can conclude that His-199 is essential for both active forms of C2,3O (EH$_2$ and EH; see Fig. 3) in its deprotonated form. This finding may be suggestive of a proton acceptor role for His-199.

As for His-246, two different substitutions were made, H246N and H246A. H246N was designed based on the hypothesis that an asparagine residue could still bind the backbone of Ile-291 through an H-bond (Fig. 1), whereas this H-bond would also be lost in the case of H246A. Our data indicate that both mutations cause a decrease in the $K_m$ values and in catalytic efficiency. In fact, the $K_m$ values for the catechol mutants (H246N)- and (H246A)-C2,3O are 470- and 3000-fold higher, respectively, than the $K_m$ value of the wild-type enzyme. Based on the structures available for two DHBDSs (8, 11), we can hypothesize that the ring of His-246 could provide a rigid surface for positioning the catechol molecule bound to iron (Fig. 1, A and B). This surface would be reduced in (H246N)-C2,3O and be completely removed in (H246A)-C2,3O, thus increasing the $K_m$ values of the two mutants. On the other hand, both mutations at position 246 cause a decrease in the $k_{cat}$ values and a surprising change in their pH dependence. In fact, the activity of H246N is almost completely abolished at a pH <7, whereas a narrow new peak is present at pH 8 with a pronounced shoulder at pH 9.5–10. Instead, mutant H246A activity profile shows only a large peak at alkaline pH, and its activity is further reduced with respect to H246N.

These findings indicate that His-246 contributes to determining both the maximum at pH 6 in the activity profile in its protonated form (the EH$_2$ form of the model) and the shoulder at pH 8 when deprotonated (EH form of the model). Thus, His-246 would be the residue involved in the conversion EH$_2$ ⇔ EH in the model of Fig. 3. Moreover, its contribution to catalysis is more important in its charged form at acidic pH values, given the higher $k_{cat}$ values at a pH of ~6 with respect to those at pH values of ~8.

Therefore we can conclude the following: (i) that His-246 is the major residue responsible for the unusual shape of C2,3O activity dependence from pH; and (ii) that His-246 is essential in its protonated state for determining the activity of the form EH$_2$ of C2,3O, whereas when unprotonated it triggers the activity of EH (Figs. 2 and 3).

As for mutant (Y255F)-C2,3O, the kinetic constants we determined (Table II) indicate that the hydroxyl group of Tyr-255 does not provide an important contribution to $K_m$, but that it significantly contributes to the $k_{cat}$ value of wt-C2,3O. In fact, the $k_{cat}$ value of mutant Y255F is 25-fold lower than that measured for the wild-type enzyme. Moreover, the variation of its $k_{cat}$ value on catechol with pH is very similar to that observed in the case of wt-C2,3O on the same substrate. Therefore, experimental $k_{cat}$ values measured at different pH values for this mutant were fitted to the same model developed in the case of the wild-type enzyme. The fitting procedure yielded three $K_m$ values very similar to those obtained for C2,3O (data not shown) with a $k_{cat}$ value only slightly increased (10.2 instead of 9.9). This would allow the conclusion that Tyr-255 does not give any, or gives only a minor, contribution to the decrease of the activity of the enzyme at alkaline pH values.

The above conclusions strongly support the identification of residues His-199, His-246, and Tyr-255 as catalytic residues, the functions of which are related to their ionization states. However, this finding gives no clues as to the identification of their exact roles in the catalytic mechanism, although it may be suggestive of their involvement in the extraction and/or release of a proton from/to the substrate.

To investigate this latter suggestion, the catalytic parameters and their pH dependence were measured using more acidic substrates. 3-formylcatechol (2,3-dihydroxybenzaldehyde; $K_m = 7.7$) and 4-formylcatechol (3,4-dihydroxybenzaldehyde; $K_m = 7.2$) were selected on the basis of their $k_{cat}$ values (Table II and III), which are within one order of magnitude from the $k_{cat}$ value for catechol, and their low inactivating activity (Table II). As shown in Fig. 2, B and C, the curves recorded on both substrates for the dependence of the $k_{cat}$ of wt-C2,3O from pH have a single, very large, and slightly asymmetric peak.

When the model described in Fig. 3 was used to fit the experimental data, an interesting result was obtained. Using the fitting procedure described in the supplemental material (available in the on-line version of this article), in this case the experimental curve could also be reproduced as the sum of four distinct Gaussian curves, each representing the contribution of the two different enzyme forms on the two substrates, and, hence, they should increase the efficiency of the reaction. The behavior observed can be explained by the hy-
Catechol 2,3-Dioxygenase from P. stutzeri OX1

Fig. 4. Mechanisms proposed for the reactions catalyzed by C2,3O as a function of pH. A, reactions 1 and 2 of Fig. 3. His-246 is in the cationic form and cannot accept protons. B, reaction 3 of Fig. 3. His-246 accepts a proton from the substrate and remains protonated during the catalytic cycle. C, reaction 4 of Fig. 3. The substrate binds to iron in the anionic form. His-246 is not necessary as a proton acceptor and remains neutral during the catalytic cycle.

The hypothesis that these groups also reduce catecholate reactivity toward O2 by their electron-withdrawing effect. This observation rules out the hypothesis of a nucleophilic attack of O2 on the substrate as proposed for C2,3O (10, 39) and supports an electrophilic radical attack as proposed for DHBD (8, 11).

As for the mutant H199N, its apparent $k_{cat}$ values on 3-formyl and 4-formyl catechol are −6 and 1% of the corresponding values measured for catechol (Table III). Thus, a formyl group on the aromatic ring leads to a more reduced efficiency of the catalytic process with respect to the effect measured for the wild-type enzyme. This behavior, different from that recorded in the case of H246N (see below), would suggest that the major role of residue His-199 is not to deprotonate the second hydroxyl group of the substrate but instead to activate the catechol ring and/or O2 for the reaction. On the basis of the structure of DHBD bound to the aromatic substrate and to NO (an analog of O2), Sato et al. (8) recently suggested that His-194 (homologous to His-199 in C2,3O) has at least three different roles (Fig. 1C, Steps 3 and 4) as follows: (i) abstraction of the second hydrogen atom from the aromatic substrate; (ii) stabilization of the partially reduced oxygen intermediales to promote the oxidative attack on the aromatic substrate; and (iii) release of a proton in the late stages of the reaction. The structural data of manganese-dependent and iron-dependent HPCD also suggest these different roles (12). Our data, excluding the fact that the acid-base catalysis is the primary role of His-199, might indirectly support the involvement of this residue in the activation of oxygen for the attack on the aromatic ring.

On the other hand, the behavior of the mutant (H246N)-C2,3O is very different, with a $k_{cat}$ value on 3-formylcatechol 4-fold higher than that on catechol (Table III). Moreover, the $k_{cat}$ value on 4-formylcatechol is only 30% of that on catechol. This value is to be confronted with the 10% reduction measured in the case of wt-C2,3O (Table III). Several chemical and structural studies indicate that the initial deprotonation of catechol is the first crucial step in extradiol cleavage reactions (8, 11, 14–16) and that all proposed mechanisms start from catechol ionization (8, 10, 11). A formyl group at position 3 favors spontaneous deprotonation of the substrate, hence compensating the reduced ability of the mutant enzyme to induce its ionization. Based on this consideration, the increase in the $k_{cat}$ value of the H246N mutant on 3-formylcatechol with respect to that of wt-C2,3O is a strong indication that His-246 is directly involved in this step of catalysis. On the other hand, the effect measured with 4-formylcatechol would suggest that the reduced substrate reactivity toward O2 mediated by the formyl electron-withdrawing effect likely prevails on the facilitated deprotonation of catechol, leading to a reduction in the $k_{cat}$, although less pronounced than that measured in the case of wt-C2,3O. Thus, our conclusion agrees with the hypothesis advanced by Vaillancourt et al. (11) that His-241 in DHBD, homologous to His-246 in P. stutzeri C2,3O, is involved in substrate deprotonation, likely in its deprotonated form at pH 7.5 (Fig. 4B).

It should be noted that we have already provided evidence that His-246 is also essential in its protonated state. An interesting possibility also exists that positively charged His-246, together with catalytic Fe$^{3+}$, both contribute to the deprotonation of the substrate, the aromatic ring of which rests on the imidazole ring of His-246 as discussed above, by lowering its $pK_a$, hence facilitating its deprotonation (Fig. 4A). The proton acceptor could be a solvent molecule bound to Fe$^{3+}$ (Fig. 4A) as initially proposed by Kita et al. (10) and more recently by Vetting et al. (12).

Finally, the $k_{cat}$ values on formylcatechols of mutant (Y255F)-C2,3O indicate that the increase in substrate acidity does not compensate for the loss of the hydroxyl group at position 255. Therefore, unlike His-246, Tyr-255 does not seem to be directly involved in substrate deprotonation. The role of the hydroxyl group could be that of hindering the negative oxygen of catecholate (Figs. 1A and 4), thus stabilizing the EH$_2$S$^{-}$ complex. Moreover, the hypothesis can be advanced that Tyr-255 and His-199 play the role of inducing asymmetry in catechol through hydrogen bonds, thus favoring specific deprotonation of the hydroxyl group, which is on the same side of Tyr-255 (Figs. 1A and 4). Instead, His-246 would be more directly involved in proton abstraction.
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The Role of the Conserved Residues His-246, His-199, and Tyr-255 in the Catalysis of Catechol 2,3-Dioxygenase from *Pseudomonas stutzeri* OX1
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