O_2 Activation

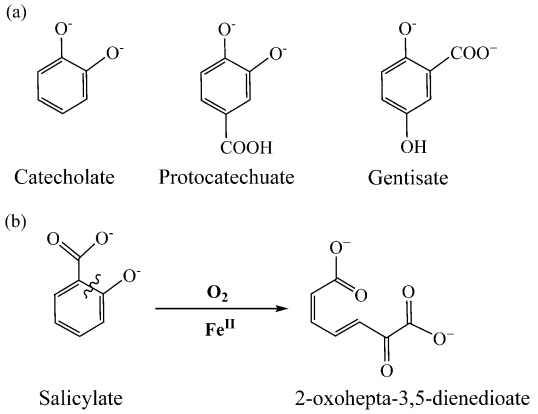
International Edition: DOI: 10.1002/anie.201506363
German Edition: DOI: 10.1002/ange.201506363

Synergistic Substrate and Oxygen Activation in Salicylate Dioxygenase Revealed by QM/MM Simulations

*Subhendu Roy and Johannes Kästner**

Abstract: *Salicylate 1,2-dioxygenase (SDO) is the first enzyme to be discovered to catalyze the oxidative cleavage of a monohydroxylated aromatic compound, namely salicylate, instead of the well-known electron-rich substrates. We have investigated the mechanism of dioxygen activation in SDO by QM/MM calculations. Our study reveals that the non-heme Fe^{II} center in SDO activates salicylate and O_2 synergistically through a strong covalent interaction to facilitate the reductive cleavage of O_2. A covalent salicylate–Fe^{II}–O_2 complex is the reactive oxygen species in this case, and its electronic structure is best described as being between the two limiting cases, Fe^{II}–O_2 and Fe^{II}–$O_2^{\bullet-}$, with partial electron transfer from the activated salicylate to O_2 via the Fe center. Thus SDO employs a synergistic strategy of substrate and oxygen activation to carry out the catalytic reaction, which is unprecedented in the family of iron dioxygenases. Moreover, O_2 activation in SDO happens without the assistance of a proton source. Our study essentially shows a new mechanistic possibility for O_2 activation.*

Mononuclear non-heme iron enzymes unlock the great oxidizing potential of dioxygen to perform a broad range of biological functions.[1–6] Iron dioxygenases that catalyze the chemically intriguing reaction of triplet O_2 with singlet organic substrates are of particular importance. The reduction potential of Fe^{II}/Fe^{III} is not favorable to activate O_2 in a one-electron reduction process.[7] Generally, most non-heme oxygenase enzymes, such as the extradiol dioxygenases, activate O_2 via a high-spin Fe^{II} that can undergo a change in redox state (Fe^{II}/Fe^{III}) in the presence of a substrate or an additional cofactor that supplies the required electrons. The other major class of enzymes, intradiol dioxygenases, use Fe^{III} where O_2 directly attacks the substrate. In essence, extradiol enzymes (Fe^{II}) use the oxygen activation pathway, whereas intradiol enzymes (Fe^{III}) follow a substrate activation mechanism.[7,8] In all cases (intradiol and extradiol), the substrates, such as substituted catechols, protocatechuates, and gentisates, are electron-rich (Scheme 1 a), thereby facilitating the electron donation that is essential for the reductive cleavage of O_2. The substrates for these dioxygenases thus are either dihydroxy compounds or contain at least one electron-donating group in the aromatic ring.[9] Various intermediates, such as Fe^{III}–$O_2^{\bullet-}$[10] or a Fe^{III}–$O_2^{\bullet-}$/$SQ^{\bullet}$–Fe^{II}–$O_2^{\bullet-}$ hybrid state (SQ = semiquinone),[11] have been proposed to be the reactive oxygen species in homoprotocatechuate 2,3-dioxygenase (HPCD) based on DFT or QM/MM calculations, respectively. Recently, salicylate 1,2-dioxygenase (SDO) was isolated from *Pseudaminobacter salicylatoxidans* and shown to catalyze the oxidative cleavage of salicylate to 2-oxohepta-3,5-dienedioic acid by a novel ring-fission mechanism (Scheme 1 b).[12] SDO plays a crucial role in the biodegradation of aromatic compounds, which are accumulated in the environment by industrial and agricultural pollution.



Scheme 1. a) Well-known substrates of dioxygenase enzymes. b) Reaction catalyzed by salicylate 1,2-dioxygenase (SDO).

Most importantly, SDO is the first enzyme to be discovered to cleave a monohydroxylated aromatic compound, namely salicylate, instead of the well-known electron-rich substrates. Even salicylate 1-monooxygenase requires the presence of an additional cofactor, FAD, for its catalytic activity.[13] SDO is extraordinary as it cleaves a substrate that is not a dihydroxy compound and does not have an electron-donating group even in the absence of a suitable cofactor. It strongly challenges the accepted idea that the substrate must supply the electrons required for O_2 cleavage in view of the unfavorable reduction potential of Fe^{II}/Fe^{III}. Therefore, the crucial question is how the O_2 activation occurs with such a monohydroxylated substrate in SDO. Another intriguing issue is the unclear role of the His162 and Arg127 residues, which have been suggested to be the active acid/base residues in SDO.[12a]

Such residues are suggested to assist in O_2 activation by stabilizing the formation of the superoxide species and/or by protonating it to produce a hydroperoxo species.[5] How do these residues assist in O_2 activation in SDO? Does O_2

[*] Dr. S. Roy, Prof. Dr. J. Kästner
Institute for Theoretical Chemistry
University of Stuttgart
Pfaffenwaldring 55, 70569 Stuttgart (Germany)
E-mail: kaestner@theochem.uni-stuttgart.de

Supporting information and ORCID(s) from the author(s) for this article are available on the WWW under http://dx.doi.org/10.1002/anie.201506363.

activation occur without a proton source? To provide answers to these fundamental queries, the chemical reactions need to be simulated in a realistic environment. A combination of quantum mechanics and molecular mechanics (QM/MM)[14] is a very promising method for simulating such enzymatic reactions in realistic environments.[15]

With an eye to answer these questions, we investigated the mechanism of dioxygen activation in SDO by combined QM/MM simulations. To the best of our knowledge, this is the first QM/MM study on a class III dioxygenase (SDO), whereas many studies have been reported for both class I (intradiol) and class II (extradiol) dioxygenases. Herein, we report an unprecedented way of molecular O_2 activation in the family of iron dioxygenases.

The enzyme SDO was obtained from the PDB structure 3NJZ,[12b] which contains SDO from the bacterium *Pseudaminobacter salicylatoxidans* with the salicylate substrate at 2.1 Å resolution. O_2 is added to the iron center of the monomer model to create an Fe–O_2 adduct in the catalytic cycle of SDO. The QM/MM calculations were carried out with the Chemshell[16] suite. The CHARMM22[17] force field was used for the MM part while the QM calculations were done with TURBOMOLE version 6.4.[18] The QM part consists of Fe, salicylate, O_2, the side chains of the three first-coordination-sphere residues His119, His121, His160, and also Arg127 (Figure 1) unless stated otherwise. The QM

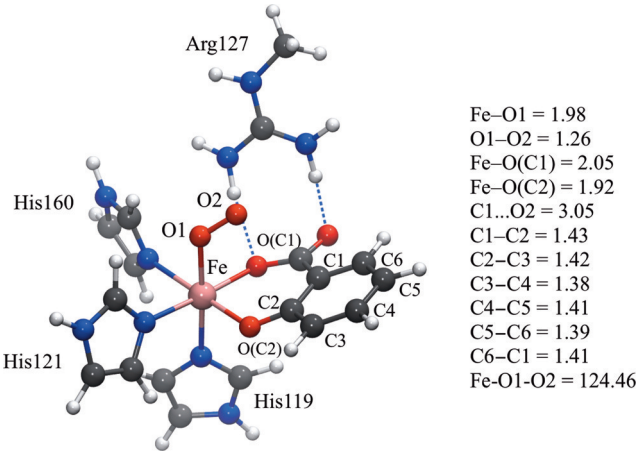


Figure 1. Optimized geometry of the ESO_2 complex **1** at the B3LYP/def2-SVP/MM level of theory. Distances are given in Å and angles in ° (C gray, H white, N blue, O red).

part is described by the hybrid B3LYP[19] functional in combination with the def2-SVP[20] basis set, a combination that has been shown to give reliable results for dioxygenases.[8,10a,11] Single-point energy calculations were performed with a larger def2-TZVP[21] basis set at the B3LYP/def2-SVP optimized geometries. The QM part carries a charge of +1 with a spin multiplicity of 5 in the geometry optimizations. The natural spin densities[22] for the complexes were calculated using the TURBOMOLE package. Furthermore, the nature of bonding of the QM region of the QM/MM optimized geometry was analyzed by natural bond orbital (NBO) analysis, as implemented in Gaussian 09.[23]

We started by analyzing the electronic structure of the enzyme–substrate–O_2 (ESO_2) complex **1** in detail to ascertain the reactive oxygen species in SDO. The coupling of quintet Fe^{II} with triplet O_2 can result in septet, quintet, and triplet spin states. The triplet spin state is 3.4 kcal mol^{-1} higher in energy than the quintet state. Whereas the septet state was calculated to be 2.7 kcal mol^{-1} lower in energy than the quintet state, the reaction barrier for the first step of the septet spin state lies at least 11.5 kcal mol^{-1} above the corresponding step on the quintet surface.[10a] Therefore, we took the quintet state of **1** as the catalytically relevant state for reactions of the mononuclear non-heme enzymes.[10a,11] The optimized ESO_2 complex **1** adopts a distorted octahedral structure, with O_2 binding to the Fe center in an end-on fashion (Figure 1). The salicylate ring is tilted towards the O2 atom of O_2 to facilitate O_2 attack. Several attempts to obtain a side-on-bound O_2 complex failed as they always resulted in an Fe–O_2 end-on complex. The Fe–O1 (O_2) bond length is 1.98 Å in **1** (Figure 1), which is shorter than the Fe–O(O_2) bond lengths (2.10–2.22 Å)[10a,11] in HPCD. The O–O bond length in bound O_2 (1.26 Å) is elongated compared to that in free O_2 (1.20 Å). The Fe–O(C1) and Fe–O(C2) bond lengths with the salicylate are 2.05 Å and 1.92 Å, respectively, which are relatively short compared to the Fe–$O_{substrate}$ bond lengths in HPCD.[11] The shorter Fe–$O_{substrate}$ bond lengths are indicative of strong metal–salicylate binding in **1**.

The important singly occupied frontier molecular orbitals (MOs) of **1** are shown in Figure 2. The highest occupied MO (HOMO, β) and the third highest (HOMO–2, β) involve significant interactions between the metal, O_2, and the salicylate. The five unoccupied β orbitals have larger metal d orbital contributions (Supporting Information, Figure S2). The occupied α Fe d orbitals are greatly stabilized. This suggests that the covalent interaction is unevenly distributed among the three moieties. Thus we see that the frontier orbitals have relatively small contributions from the metal d orbitals.

In **1**, the natural spin densities on Fe, O_2, and Sal (Sal = salicylate) are 3.80, −0.27, and 0.29, respectively (Table S1), which implies antiferromagnetic coupling between the Fe and O_2 centers with partial spin density on the distal O2 atom. The spin density on the substrate is largely localized on the O donor atoms. The comparatively smaller spin density on the Fe center is attributed to the strong covalent interactions with salicylate and O_2, which reduce the metal center. Salicylate retains a planar structure in **1**, which strongly defers any chance of disruption of the planar aromaticity to acquire radical character (as is also supported by the spin density) by transferring one electron to O_2. How is O_2 activated? The answer lies in the strongly covalent nature of **1** as revealed by the MOs (Figure 2). In this case, the Fe^{II} center activates the salicylate through a strong covalent interaction to transfer partial β electron density to the O_2 π* orbitals via the Fe^{II} center. The additional electron density in O_2 resides in the π_{op}* orbitals, where the in-plane (ip) and out-of-plane (op) orbitals are defined by the Fe-O-O plane. The C–C π-bonds in salicylate are perturbed by a strong covalent interaction with the Fe center, as shown by the HOMO (β) and HOMO–2 (β). The elongation of the C1–C2 bond (1.43 Å) compared

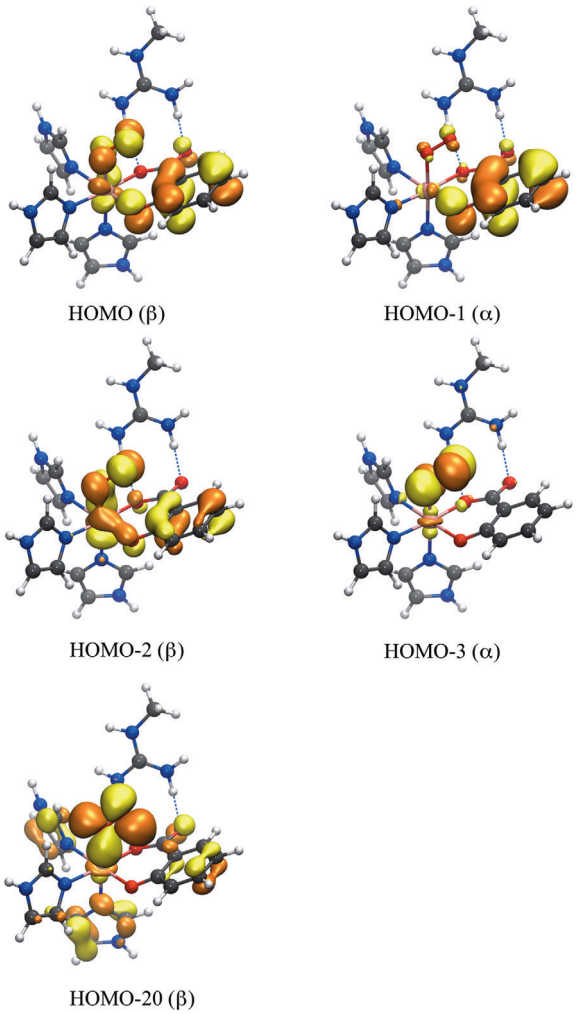
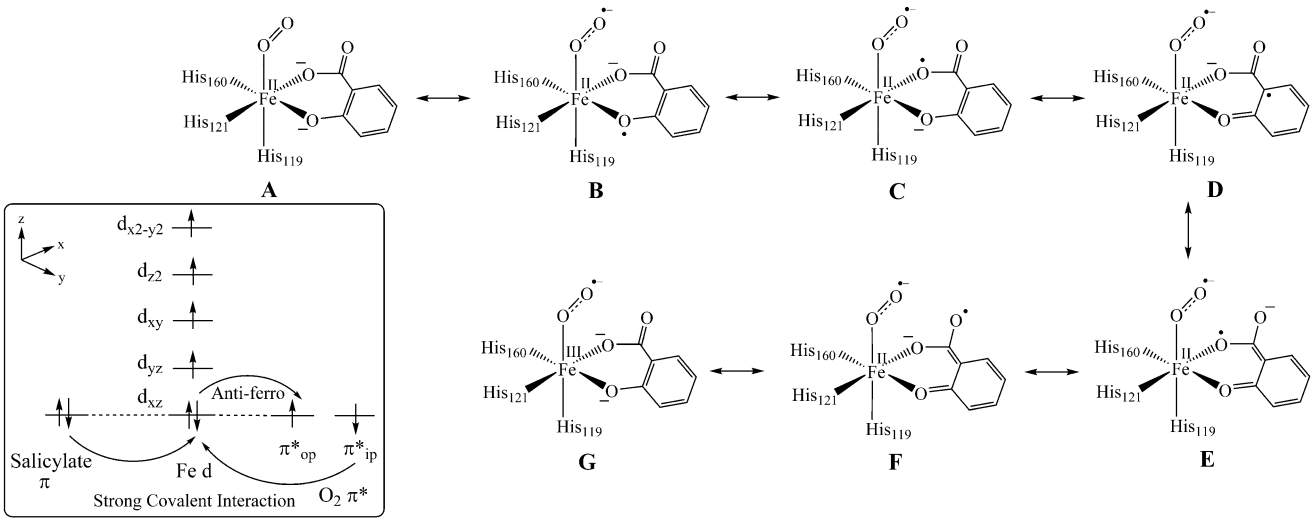


Figure 2. Important singly occupied molecular orbitals of the ESO_2 complex **1**.

to the other C–C bonds (Figure 1) in the ring is noteworthy in this respect. This implies that the salicylate and the O_2 are synergistically activated for reaction by the Fe^{II} center. Even the relatively close contact between the O2 and C1 atoms (3.05 Å) in **1** hints at the start of the electron-transfer process from the activated C1–C2 π bond to the O_2, which is corroborated by HOMO−1 (α) and HOMO−20 (β).

The seven possible resonance structures of **1** are shown in Scheme 2. Apart from the in-plane σ bonding between the Fe center and salicylate, NBO analysis revealed that the β electron on the O(C2) atom of salicylate is involved in a strong, π-covalent donor interaction with the Fe center with 0.886 O(C2)(p)+0.114 Fe(d) composition with 89.9% occupancy, which is reflected in the multiple-bond character of the Fe–O(C2) bond (Table S2, Figure S4). It results in partial α spin density on the O(C2) atom. The involvement of salicylate ring π orbitals in the π-covalent donor interactions is clearly evident from the HOMO (β) and HOMO−2 (β). Similar strong covalent interactions to transfer partial β electron density from the substrate to O_2 are observed in the intradiol dioxygenases.[24] In this case, the strong π-covalent donor interactions play a vital role in the bonding of **1**. The C2–O(C2) bond has single-bond nature, which rules out any semiquinone-like structures. The antiferromagnetic coupling between the O_2 and Fe centers transfers β electron density from the Fe d_{xz} orbital to the O_2 π_{op}^* orbital, leading to partial superoxide character. The partial transfer of β electron density from the occupied O_2 π* orbitals to the unoccupied Fe β d orbitals reflects the covalent interaction between O_2 and Fe. Thus the Fe center acquires β electron density from the salicylate through a strong covalent interaction to pass it on to O_2 for its activation (Scheme 2, inset).[24,25] The sums of the NBO charges of salicylate and O_2 are −1.51 e and −0.44 e, respectively. These values suggest that the salicylate donates 0.49 e, and that the O_2 receives 0.44 e, which provides further support for the partial electron transfer from the activated salicylate to O_2 via the Fe center. Therefore, based on the structural parameters, the MOs, NBO analysis, and the spin densities, the resonance structures **A** and **B** are the major contributors for describing **1**. Herein, we thus propose that a covalent Sal–Fe^{II}–O_2 complex is the reactive oxygen species



Scheme 2. Possible resonance structures for **1** and electron-transfer process in **1** (inset).

in SDO. The electronic nature of the Fe–O_2 adduct is best described as being between the two limiting cases, Fe^{II}–O_2 and Fe^{II}–$O_2^{\bullet-}$, with partial electron transfer from the activated salicylate via the Fe center to activate the O_2 for reaction. The unchanged Fe^{II} oxidation state in the reactive species draws strong support from the fact that the activity of the dioxygenase enzymes does not depend on the redox potential of the metal atoms.[25,26]

Our analysis reveals that groups that enhance the electron-donating ability of the salicylate (such as OH and NH_2 groups) should facilitate the activation of O_2 and thereby increase the catalytic efficiency of SDO. Indeed, this proposition is in good agreement with experimental observations.[12a] Similarly, it would be interesting to study the effect of electron-withdrawing groups such as F and NO_2 on the salicylate ring on the catalytic activity of SDO.

SDO is unique as substrate activation is done by a reduced Fe^{II} metal ion in contrast to an oxidized Fe^{III} ion in the intradiol dioxygenases. We also looked at the enzyme substrate complex without O_2, which features a strong covalent interaction between metal and salicylate as evident from the Fe–O(C1) and Fe–O(C2) bond lengths (2.08 Å and 1.92 Å, respectively, compared to 2.05 Å and 1.92 Å, respectively, in the presence of O_2). Our study establishes that SDO employs a unique strategy of synergistic substrate and oxygen activation to carry out the oxidative cleavage of a monohydroxylated aromatic compound such as salicylate, which is unprecedented for the family of iron dioxygenases. Indeed, the transition state **TS1** of the first step to form the alkylperoxo intermediate[27,28] **2** (Figure 3) shows the full formation of the O2–C1 bond with simultaneous transfer of two electrons from the π system of salicylate to O_2 (see the Supporting Information).

In **1**, His162 is positioned too far away (6.4 Å) from O_2 to act as an active acid/base residue. We could not even locate any water molecules that act as a proton source after several attempts of MD simulations of the solvated enzyme. Therefore, there is no proton source for the catalytic activity of SDO. This is highly unusual compared to other mononuclear non-heme iron dioxygenases.[5] Instead, we observed that Arg127 stabilizes the carboxylate O atoms of the salicylate in **1** through hydrogen bonding. Arg residues were reported to assist in O_2 activation in 3-hydroxyanthranilate 3,4-dioxygenase[5] and in AlkB repair enzymes.[29] Arg83 is located opposite to the dioxygen-binding site. Our results are in excellent agreement with the experimental observations, where Arg127 was shown to be crucial for the enzymatic activity of SDO based on mutational studies.[30] In this context, we suggest to replace Arg127 by a familiar proton source such as His in SDO to observe the effect of this replacement on the catalytic efficiency of SDO in cleaving monohydroxylated aromatic substrates.

We investigated the effect exerted by the protein environment on the ESO_2 complex by comparison to a gas-phase model. This model comprises the QM region of the full QM/MM simulations with the positions of the H atoms fixed at the truncation point. Gas-phase optimization on the model resulted in the spontaneous rotation of O_2 around the Fe–O bond so that O_2 points towards the His ligands (Figure S3); this rotation can be ascribed to the absence of the protein

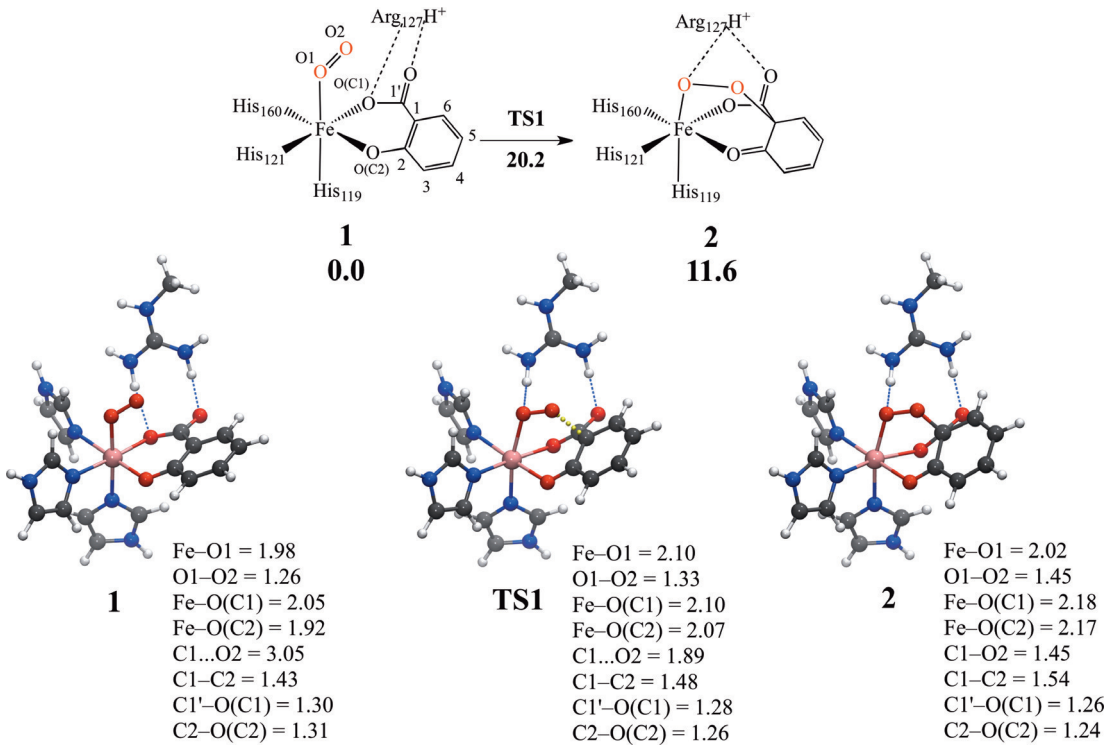


Figure 3. Optimized geometries of **1**, **TS1**, and **2** in the quintet state at the B3LYP/def2-SVP/MM level of theory (distances in Å, angles in °, and energies in kcal mol^{-1}).

environment.[10a,11] The triplet character of O_2 (Table S3) increases in the optimized gas-phase geometry along with an increase in the spin density on the Fe center (4.03). It shows the significant influence of the protein environment on the electronic structure of **1** as well as on the proper orientation of the O_2 molecule pointing towards the substrate. However, the cluster models also show significant metal–salicylate covalent interactions as already found in the full QM/MM calculations. Moreover, the 3His ligand motif facilitates the reaction by enhancing the covalent interaction between Fe and O_2 (see the Supporting Information).

In conclusion, we have shown by QM/MM simulations that the non-heme Fe^{II} center activates salicylate and O_2 synergistically by a strong covalent interaction in **1**. A covalent Sal–Fe^{II}–O_2 complex is the reactive oxygen species in the catalytic cycle of SDO, and its electronic structure is best described as being between the two limiting cases, Fe^{II}–O_2 and Fe^{II}–$O_2^{\bullet-}$, with partial electron transfer from the activated salicylate to O_2 via the Fe center. SDO employs a unique strategy of synergistic substrate and oxygen activation to carry out the reductive cleavage of O_2, which is unprecedented in the family of iron dioxygenases. Moreover, O_2 activation in SDO happens without the assistance of a proton source, and the protein environment significantly affects the electronic structure of **1** to facilitate the reaction. We feel that these insights will be greatly beneficial for designing biomimetic catalysts for utilizing O_2, especially in the context of substrate selection.

Acknowledgements

This work was financially supported by the German Research Foundation (DFG) within the collaborative research center SFB716 as well as by the Baden-Württemberg Stiftung.

Keywords: dioxygen · dioxygenases · metalloenzymes · non-heme iron proteins · QM/MM simulations

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 1168–1172
Angew. Chem. **2016**, *128*, 1182–1187

[1] E. G. Kovaleva, J. D. Lipscomb, *Nat. Chem. Tech.* **2008**, *4*, 186.
[2] M. Costas, M. P. Mehn, M. P. Jensen, L. Que, Jr., *Chem. Rev.* **2004**, *104*, 939.
[3] D. Buongiorno, G. D. Straganz, *Coord. Chem. Rev.* **2013**, *257*, 541.
[4] E. I. Solomon, T. C. Brunold, M. I. Davis, J. N. Kemsley, S. K. Lee, N. Lehnert, F. Neese, A. J. Skulan, Y. S. Yang, J. Zhou, *Chem. Rev.* **2000**, *100*, 235.
[5] T. D. H. Bugg, S. Ramaswamy, *Curr. Opin. Chem. Biol.* **2008**, *12*, 134.
[6] E. G. Kovaleva, M. B. Neibergall, S. Chakrabarty, J. D. Lipscomb, *Acc. Chem. Res.* **2007**, *40*, 475.
[7] M. Y. M. Pau, J. D. Lipscomb, E. I. Solomon, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 18355.
[8] T. Borowski, P. E. M. Siegbahn, *J. Am. Chem. Soc.* **2006**, *128*, 12941.
[9] M. R. Harpel, J. D. Lipscomb, *J. Biol. Chem.* **1990**, *265*, 22187.
[10] a) G. J. Christian, S. F. Ye, F. Neese, *Chem. Sci.* **2012**, *3*, 1600; b) H. Hirao, K. Morokuma, *J. Am. Chem. Soc.* **2010**, *132*, 17901; c) W. A. van der Donk, C. Krebs, J. M. Bollinger, Jr., *Curr. Opin. Struct. Biol.* **2010**, *20*, 673.
[11] G. Dong, S. Shaik, W. Z. Lai, *Chem. Sci.* **2013**, *4*, 3624.
[12] a) I. Matera, M. Ferraroni, S. Bürger, A. Scozzafava, A. Stolz, F. Briganti, *J. Mol. Biol.* **2008**, *380*, 856; b) M. Ferraroni, I. Matera, L. Steimer, S. Bürger, A. Scozzafava, A. Stolz, F. Briganti, *J. Struct. Biol.* **2012**, *177*, 431; c) M. Ferraroni, I. Matera, S. Bürger, S. Reichert, L. Steimer, A. Scozzafava, A. Stolz, F. Briganti, *FEBS J.* **2013**, *280*, 1643.
[13] S. Yamamoto, M. Katagiri, H. Maeno, O. Hayaishi, *J. Biol. Chem.* **1965**, *240*, 3408.
[14] a) A. Warshel, M. Karplus, *J. Am. Chem. Soc.* **1972**, *94*, 5612; b) A. Warshel, M. Levitt, *J. Mol. Biol.* **1976**, *103*, 227.
[15] a) H. Senn, W. Thiel, *Topics in Current Chemistry*, Vol. 268, Springer, Berlin, **2007**, p. 173; b) H. Senn, W. Thiel, *Curr. Opin. Chem. Biol.* **2007**, *11*, 182; c) H. Lin, D. Truhlar, *Theor. Chem. Acc.* **2007**, *117*, 185.
[16] a) P. Sherwood et al., *J. Mol. Struct. (Theochem.)* **2003**, *632*, 1; b) S. Metz, J. Kästner, A. A. Sokol, T. W. Keal, P. Sherwood, *WIREs Comput. Mol. Sci.* **2014**, *4*, 101.
[17] a) A. D. MacKerell, Jr. et al., *J. Phys. Chem. B* **1998**, *102*, 3586; b) A. D. MacKerell, Jr., M. Feig, C. L. Brooks III, *J. Comput. Chem.* **2004**, *25*, 1400; c) N. Foloppe, A. D. MacKerell, Jr., *J. Comput. Chem.* **2000**, *21*, 86; d) A. D. MacKerell, Jr., N. Banavali, *J. Comput. Chem.* **2000**, *21*, 105.
[18] *TURBOMOLE*, V6.4 2012, a development of the University of Karlsruhe and Forschungszentrum Karlsruhe GmbH, 1989–2007, TURBOMOLE GmbH, since **2007**; available from www.turbomole.com.
[19] a) A. D. Becke, *Phys. Rev. A* **1988**, *38*, 3098; b) C. Lee, W. Yang, R. G. Parr, *Phys. Rev. B* **1988**, *37*, 785; c) A. D. Becke, *J. Chem. Phys.* **1993**, *98*, 5648.
[20] A. Schäfer, H. Horn, R. Ahlrichs, *J. Chem. Phys.* **1992**, *97*, 2571.
[21] A. Schäfer, C. Huber, R. Ahlrichs, *J. Chem. Phys.* **1994**, *100*, 5829.
[22] A. E. Reed, L. A. Curtiss, F. Weinhold, *Chem. Rev.* **1988**, *88*, 899.
[23] Gaussian 09, Revision D.01, M. J. Frisch et al., Gaussian, Inc., Wallingford CT, **2013**. See the Supporting Information for details.
[24] M. Y. M. Pau, M. I. Davis, A. M. Orville, J. D. Lipscomb, E. I. Solomon, *J. Am. Chem. Soc.* **2007**, *129*, 1944.
[25] M. M. Mbughuni, M. Chakrabarti, J. A. Hayden, K. K. Meier, J. J. Dalluge, M. P. Hendrich, E. Münck, J. D. Lipscomb, *Biochemistry* **2011**, *50*, 10262.
[26] a) J. P. Emerson, E. G. Kovaleva, E. R. Farquhar, J. D. Lipscomb, L. Que, Jr., *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 7347; b) M. M. Mbughuni, M. Chakrabarti, J. A. Hayden, E. L. Bominaar, M. P. Hendrich, E. Munck, J. D. Lipscomb, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 16788.
[27] E. G. Kovaleva, J. D. Lipscomb, *Science* **2007**, *316*, 453.
[28] J.-H. Jeounga, M. Bommera, T.-Y. Lina, H. Dobbeka, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 12625.
[29] M. G. Quesne, R. Latifi, L. E. Gonzalez-Ovalle, D. Kumar, S. P. de Visser, *Chem. Eur. J.* **2014**, *20*, 435.
[30] E. Eppinger, M. Ferraroni, S. Bürger, L. Steimer, G. Peng, F. Briganti, A. Stolz, *Biochim. Biophys. Acta Proteins Proteomics* **2015**, *1854*, 1425.

Received: July 10, 2015
Revised: September 18, 2015
Published online: November 24, 2015

