Multiple pathways guide oxygen diffusion into flavoenzyme active sites

Baron, Riccardo; Riley, Conor; Chenprakhon, Pirom; Thotsaporn, Kittisak; Winter, Remko T.; Alfieri, Andrea; Forneris, Federico; van Berkel, Willem J. H.; Chaiyen, Pimchai; Fraaije, Marco

Published in:
Proceedings of the National Academy of Sciences of the United States of America

DOI:
10.1073/pnas.0903809106

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2009

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
10.1073/pnas.0903809106

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Multiple pathways guide oxygen diffusion into flavoenzyme active sites

Riccardo Baronb,1, Conor Rileya,2, Pirom Chenprakhonbd,2, Kittisak Thotsapornc,2, Remko T. Wintera,2,3, Andrea Alfieri5, Federico Formeri3, Willem J. H. van Berkel5, Pimchai Chaiyenc, Marco W. Fraajie, Andrea Mattevi5,1, and J. Andrew McCammona,2,6,1

Department of Chemistry and Biochemistry and 2Pharmacology, 3Center for Theoretical Biological Physics, and 4Howard Hughes Medical Institute, University of California at San Diego, La Jolla, CA 92093-0365; Department of Biochemistry and Center for Excellence in Protein Structure and Function, Faculty of Science, and 5Institute for Innovative Learning, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand; 6Laboratory of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands; 7Department of Genetics and Microbiology, University of Pavia, Via Ferrata 1, 27100 Pavia, Italy; and 8Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

Edited by William A. Eaton, National Institutes of Health, Bethesda, MD, and approved April 29, 2009 (received for review April 6, 2009)

Dioxygen (O2) and other gas molecules have a fundamental role in a variety of enzymatic reactions. However, it is only poorly understood which O2 uptake mechanism enzymes employ to promote efficient catalysis and how general this is. We investigated O2 diffusion pathways into monooxygenase and oxidase flavoenzymes, using an integrated computational and experimental approach. Enhanced-statistics molecular dynamics simulations reveal spontaneous protein-guided O2 diffusion from the bulk solvent to preorganized protein cavities. The predicted protein-guided diffusion paths and the importance of key cavity residues for oxygen diffusion were verified by combining site-directed mutagenesis, rapid kinetics experiments, and high-resolution X-ray structures. This study indicates that monooxygenase and oxidase flavoenzymes employ multiple funnel-shaped diffusion pathways to absorb O2 from the solvent and direct it to the reacting C4a atom of the flavin cofactor. The differences in O2 reactivity among dehydrogenases, monooxygenases, and oxidases ultimately resides in the fine modulation of the local environment embedding the reactive locus of the flavin.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freedly available online through the PNAS open access option.

1To whom correspondence should be addressed. E-mail: rbaron@mcsorlan.ucsd.edu or mattevi@psigen.univip.it.
2C.R. and P. Chenprakhon contributed equally to this work.
3K.T. and R.T.W. contributed equally to this work.

This article contains supporting information online at www.pnas.org/cgi/content/full/0903809106/DCSupplemental.

PNAS | June 30, 2009 | vol. 106 | no. 26 | 10603–10608

www.pnas.org/cgi/10.1073/pnas.0903809106
Out of a total of 500 O2 trajectories at 300 K, we observe 4 conduct O2 molecules (red spheres) (observed from simulations at 300 K (volume isosurfaces) complete spontaneous diffusion pathways that bring O2 molecules in front of the re-side of FMNH− (Fig. 1) starting from random configurations in the bulk solvent. Successful paths typically cover overall distances of ~40–60 Å from the protein surface to the flavin C4a carbon and display a stepwise behavior from the time of entrance through the C2 protein surface (Fig. 1B). In fact, O2 molecules may temporarily reside on the surface (e.g., Fig. 1B, step A) and generally visit several cavities along each of these paths (e.g., Fig. 1B, steps B and C). An example path is depicted in Fig. 2 and available as Movie S1 video online. Additionally, trajectories show O2 molecules residing transiently in cavities and niches on the protein surface characterized by the presence of Ala and/or Ile hydrophobic residues, an ideal physicochemical environment to host O2 molecules. These additional paths carry O2 molecules inside the protein, but not in proximity of the C4a flavin atom (distance >6 Å) were therefore distinguished from the above mentioned “complete paths” (see also SI Text). 350 K simulations confirm the location of these paths (15 and 25 corresponding events, respectively; data not shown). The relatively limited number of complete paths observed might be, in part, dependent on the 30-ns overall sampling considered (13% of O2 molecules are inside the protein matrix at the end of our runs at 300 K; 27% at 350 K). However, we stress that this sampling time was sufficient to capture a multitude of diffusion paths.

The observed spontaneous O2 diffusion matches a model consisting of multiple diffusion paths converging toward residues Phe-270 and Phe-266, in front of the re-side of the reduced FMNH− cofactor (Fig. 3 and Scheme 1). Once O2 molecules reach Phe-266 and Phe-270 (Fig. 1B, step C), transient fluctuations of these aromatic side chains allow O2 to further diffuse into a smaller cavity defined by flavin C4a, His-396, Phe-266, and Phe-270 (Fig. 1B, step D). The side chain of His-396 was proposed to facilitate formation of the C4a-hydroperoxide intermediate (31) and our MD simulations are consistent with the hypothesized role of this preorganized oxygen cavity for C4a-intermediate stabilization. Moreover, they also show that this preorganized cavity is characterized by remarkable side chain

---

**Fig. 1.** O2 spontaneous diffusion into C2. (A) Four complete paths are observed from simulations at 300 K (volume isosurfaces) (Left). All paths conduct O2 molecules (red spheres) (Right) to the re-side of the FMNH (reduced flavin cofactor, green sticks) in close contact with its C4a carbon (Right). (B) Time series of the O2 − C4a distance for the successful diffusion paths and key steps (see text). The dashed horizontal line defines the C4a flavin oxygen cavity.
flexibility, a prerequisite of O$_2$ diffusion toward the active site (Fig. 2).

Computational predictions and our model for protein-guided O$_2$ diffusion were validated by combining site-directed mutagenesis and rapid kinetics experiments. Reactions of C$_2$-FMNH$^-$/O$_2$ to form a C4a-hydroperoxyflavin intermediate were monitored using a stopped-flow spectrophotometer (Figs. S2–S4). The kinetics were comparatively investigated in the wild-type protein (35) and in 4 mutants that target Phe-266, the residue suggested by the MD simulations to represent the entry point for the O$_2$ access to the reactive C4a locus of the flavin ring. The main results of this kinetic analysis were the following (Table 1): (i) Binding of the p-hydroxyphenylacetate (HPA) substrate to the wild-type C$_2$-FMNH$^-$ complex causes a 20-fold reduction in the rate of formation of the C4a-hydroperoxyflavin intermediate; (ii) mutation of Phe-266 with a bulky Trp side chain causes a very similar drop in rate of the oxygen reaction; and (iii) replacement of Phe-266 with smaller side chains (Phe266Pro, Phe266Gly, and Phe266Ala) has little (<5-fold) effect on C4a-hydroperoxyflavin formation, indicating that these mutants retain the ability to efficiently react with oxygen. Correlating the kinetics with the mechanism of oxygen diffusion is especially difficult because the measured rates of the oxygen reaction and C4a-hydroperoxyflavin formation are composite parameters that result from the rates of oxygen diffusion through the protein matrix and of the chemical steps along the electron-transfer reaction between the oxygen and reduced flavin. Bearing in mind this inherent difficulty in data interpretation, we envision the following working model. O$_2$ diffusion trajectories converge to Phe-266, at the entrance of the oxygen cavity in front of the flavin C4a atom (Figs. 1 and 2). As shown by the crystal structures, HPA binding involves a conformational change of the flavin C4a atom (Figs. 1 and 2). As shown by the crystal structures, HPA binding involves a conformational change of the flavin C4a atom (Figs. 1 and 2).

To further validate the O$_2$ diffusion model obtained from MD simulations, we constructed the C$_2$(Tyr296Phe) mutant that targets a residue close to the reactive site of the flavin whereas it is not part of the predicted diffusion paths. This mutant reacts with O$_2$ identically to the wild-type enzyme; the C4a-hydroperoxyflavin is formed with rate constants of $1.2 \times 10^8$ and $4.0 \times 10^7$ M$^{-1}$s$^{-1}$ in absence and presence of HPA, respectively (Table 1). These values are essentially the same as those of the wild-type enzyme and confirm that Tyr-296 is not part of any O$_2$ diffusion path as predicted by our simulations.

**Oxygen Diffusion into AldO.** O$_2$ diffusion into an oxidase was addressed for AldO, a soluble monomeric flavoprotein of the class of vanillyl-alcohol oxidase, which contains a covalently bound FAD cofactor (32, 37). AldO catalyzes a typical 2-electron flavin-mediated oxidation of a terminal C-OH moiety of a polyol substrate to the corresponding aldehyde, with the concomitant reduction of the flavin cofactor. The reduced flavin (FADH$^-$) then reacts with O$_2$ to form H$_2$O$_2$, which completes the catalytic cycle. Because its crystal structure has been solved at high resolution (1.1 Å) (32) and the kinetic mechanism has been established by presteady state kinetic analyses (37), AldO serves as an excellent oxidase prototype.

Enhanced-statistics MD simulations were initialized based on the 3-dimensional structure of FADH$^-$-complexed AldO and extended for 50 ns of overall enhanced-time (Table S1). These simulations were set-up to capture O$_2$ diffusion before FADH$^-$ oxidation. Out of a total of 500 O$_2$ trajectories at 300 K, we observe 5 complete spontaneous diffusion pathways that bring O$_2$ molecules in front of the isoalloxazine ring of the FADH$^-$ cofactor (Fig. 4), starting from random configurations in the bulk solvent. Simulations at 350 K confirm the location of these successful paths (22 events; data not shown). Several O$_2$ molecules that initially reside in cavities on the protein surface subsequently diffuse into the AldO interior (e.g., Fig. 4B, steps A, B, D, F, and H). As observed in the C$_2$ monooxygenase system, these surface cavities typically display at least one hydrophobic residue, such as Ala and/or Ile. A remarkable example is the all-Ala pocket of Ala-88, Ala-91, and Ala-277 used to capture O$_2$ molecules from the bulk solvent (Fig. S5).

The spontaneous O$_2$ diffusion into AldO matches a model consisting of multiple diffusion paths converging toward a few key residues neighboring the reactive moiety of the flavin cofactor, similarly to what observed for C$_2$. All protein-guided diffusion pathways converge into a site defined by Ala-105, Tyr-87, and Thr-120 at ~6 Å from the re-side of FADH$^-$ (Figs. 4 and 5). Once O$_2$ molecules reach Tyr-87 or Thr-120, they (3

---

**Table 1. Reoxidation rates for reduced C$_2$(mutant)-FMNH$^-$ with O$_2$ in presence/absence of HPA**

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Rate constants of formation of C4a-hydroperoxyflavin, 10$^8$ M$^{-1}$s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$-$HPA</td>
</tr>
<tr>
<td>Wild-type</td>
<td>110</td>
</tr>
<tr>
<td>Phe266Gly*</td>
<td>15</td>
</tr>
<tr>
<td>Phe266Ala*</td>
<td>20</td>
</tr>
<tr>
<td>Phe266Pro*</td>
<td>14</td>
</tr>
<tr>
<td>Phe266Trp*</td>
<td>4.8</td>
</tr>
<tr>
<td>Tyr296Phe</td>
<td>120</td>
</tr>
</tbody>
</table>

*HPA, reaction of C$_2$(mutant)-FMNH$^-$ with O$_2$ in presence of HPA. $-$HPA, reaction of C$_2$(mutant)-FMNH$^-$ with O$_2$ in absence of HPA.

*Different from the wild-type, HPA does not affect the oxygen reactivity of the four Phe266 mutants which bind HPA only after reaction with O$_2$ and formation of C4a-hydroperoxyflavin (see Figs. S3 and S4).
paths out of 5) eventually proceed to a preorganized cavity defined by flavin C4a and Ala-105, which coincides with the cavity previously proposed based on AldO X-ray structures (32). Thus, the side chain of Ala-105 creates a favorable hydrophobic environment to stabilize the presence of an O2 molecule next to the reduced flavin (Fig. 4 A and B, steps C and G). Clearly, protein dynamics is required for the successful diffusion of O2 molecules to the active site. It is striking to observe that, similar to C2, all complete diffusion pathways (i.e., all pathways carrying oxygen from the bulk solvent to the reduced flavin cofactor) converge to only one side of the reduced flavin (re-side) (Fig. 5 and Scheme 1). Moreover, they all cross only a limited part of the AldO protein structure, i.e., the substrate-domain.

Table 2 summarizes the rapid kinetics experiments for oxygen reactivity of reduced AldO and its Ala-105 mutants. The data show that the rate constant of AldO reoxidation (13 × 10^4 M^-1 s^-1) is hardly affected by the Ala105Ser mutation (14 × 10^4 M^-1 s^-1) whereas the Ala105Gly mutant is somewhat faster (20 × 10^4 M^-1 s^-1) (Fig. S6). Apparently, small changes in the size of the side chain at position 105 have little effect on oxygen reactivity. In this regard, we notice that one would not expect a drastic increase in reactivity with oxygen in the Ala105Gly mutant, because the reaction rate of wild-type AldO is already high and is also limited by other funnel residues and the actual electron-transfer step from O2 to the flavin (3, 32). All attempts to replace Ala-105 with larger side chains failed because the mutants were extremely unstable and/or unable to bind FAD. However, strong support for the role of Ala-105 as O2 entry point to the active site is provided by recently reported mutagenesis data on plant L-galactono-1,4-lactone dehydrogenase (GALDH) (38), which shares 25% sequence identity with AldO. As a typical dehydrogenase, this enzyme reacts poorly with O2. However, its oxygen reactivity could be substantially increased (400-fold) to a level comparable to that of typical oxidases by simply mutating Ala-113 (homologous to Ala-105 of AldO) to Gly (38). Apparently, Ala-105 in AldO already allows O2 access to the reduced FAD whereas in GALDH—due to its specific active site—it needs to be replaced by a smaller residue to allow O2 to pass through. This finding indicates that, consistently with our MD predictions for AldO, also in GALDH O2 diffuses and approaches the flavin from the position corresponding to that of AldO Ala-105.

**Discussion**

We used an integrated computational and experimental procedure, combining X-ray crystallography, enhanced-statistics MD simulations, rapid kinetic experiments, and site-directed mutagenesis to shed light on how O2 molecules diffuse into the active site of flavoenzymes with different catalytic activity (monooxygenase and oxidase) and folding topologies. In both simulated systems, distinct and multiple protein-guided O2 diffusion pathways converge toward defined active site entry points, forming funnel architectures. The observation that a specific set of residues form entry sites for the access of oxygen to the flavin cofactor—as Phe-266 in C2 and Ala-105 in AldO—is consistent with what was recently reported for cholesterol oxidase (12) and sarcosine oxidase (39). However, this does not strictly imply the presence of individual diffusion pathways. Instead, our results suggest that multiple diffusion pathways, converging to a few key residues, are operative and represent a more effective structural model to combine high specificity and high kinetic efficiency in oxygen-using flavoenzymes. From a computational standpoint, we emphasize that in this work, O2 diffusion was only solvent- or protein-guided—i.e., no biasing force was used to direct the reactants to the protein active sites. We stress, therefore, that a direct picture of the diffusion process at the atomic scale was obtained a priori and only subsequently used as a prediction tool to design the experiments addressing oxygen reactivity.

Our observations are also relevant to the understanding of the reaction mechanism of oxygen with reduced flavin (3, 26). MD simulations indicate that O2 is able to reach specifically the flavin C4a atom both in the monooxygenase and the oxidase (Figs. 1 and 4). This implies that—indeed on the nature of the enzymatic reaction—O2 would react with the flavin by direct contact with the C4a atom of the cofactor. In this scenario, the C4a atom appears to be the locus directly involved in the electron transfer from the reduced cofactor to O2, which is consistent with isotope effect studies performed on glucose oxidase (29). The combination of our data with the results obtained by site-directed mutagenesis on various flavoenzymes (38, 39) indicates that the difference among dehydrogenases, monooxygenases, and oxidases ultimately resides in the fine modulation of the local environment embedding the C4a flavin.
atom; its accessibility, the distribution of charged and polar groups to favor electron transfer between reduced flavin and \( \text{O}_2 \), and the extent to which the oxygen cavity is capable of stabilizing the C4a-hydroperoxyflavin.

**Methods**

**Molecular Model and Enhanced-Statistics MD Simulations.** Ten MD trajectories of the C2 homodimer (chain A and D; 798 residues; FMNH\(^+\) cofactor; initial model PDB entry 2JBS; 2.8 Å resolution) (31) and 10 trajectories of the AldO monomer (142 residues; FADH\(^-\) cofactor; initial model PDB entry 2VFR; 1.1 Å resolution) (32) at 300 and 350 K were generated using the GROMOS software for biomolecular simulations (40), the GROMOS 53A6 parameter set (41), and compatible ion parameters (42) in the SPC water model (43). A summary is reported in **SI Text** and Table S1. A procedure for enhanced-statistics MD simulation was used for \( \text{O}_2 \) spontaneous diffusion. It involves (i) reduced masses (1.6 u) for the oxygen atoms in the \( \text{O}_2 \) molecules (21); (ii) enhanced statistics from 100 (independent and noninteracting) \( \text{O}_2 \) molecules in the system, kept by a minimum pair distance by a network of repulsive half-harmonic-distance-restraining potentials during the equilibration phase (to avoid biasing their initial location before diffusion); (iii) a fast grid-based pairwise-constructed algorithm (44) as implemented in the GROMOS05 MD++ module (40); (iv) enhanced statistics from 10 independent MD runs at 300 and 350 K. QO diffusion statistics were collected over a total of 10 independent trajectories of systems with 100 \( \text{O}_2 \) molecules each, monitored for a total enhanced-MD time of 30 (C2) or 50 (AldO) ns. The rather high \( \text{O}_2 \) apparent concentrations (C\(_{\text{O}_2}\) ≈ 0.14 M or AldO; C\(_{\text{O}_2}\) ≈ 0.3 M, for comparison, an \( \text{O}_2 \) buffer is 1.2 mM) allow capturing a sufficient statistics of \( \text{O}_2 \)-protein encounter events without perturbing protein overall structure at both 300 and 350 K. RMSD time series of C2 and AldO backbone C\(^{\text{\beta}}\)-atoms are reported in Figs. 57 and 58, together with computational details. The term “enhanced-MD time” points out that the kinetic properties of simulated \( \text{O}_2 \) molecules differ from standard MD simulation. Computer simulations were used to predict the location of oxygen diffusion pathways along C2 and AldO configurational distributions; instead, kinetic properties were measured by experiments. The term “enhanced-statistics” emphasizes that an improved statistics is achieved compared with standard MD simulation. No biasing force or potential is used to bring \( \text{O}_2 \) molecules inside the enzyme active site. Instead, \( \text{O}_2 \) freely diffuses on the free-energy landscape starting from a nonarbitrary configuration and spontaneously reaches the preorganized cavities described in the text. The Pymol (45) and VMD (46) software packages were used for graphical representations.

**Rapid Kinetics and Oxygen Reactivity.** \( \text{C}_2 \) Rapid kinetics measurements were performed at 277 K with a Hi-Tech Scientific Model SF-61DX stopped-flow spectrophotometer in single- or double-mixing mode (observation cell optical path-length of 1 cm). The stopped-flow instrument was maintained under anaerobic conditions by flushing the flow system with an \( \text{O}_2 \) scrubbing solution consisting of 400 μM glucose, 1 mg/ml \( \text{glucose oxidase} \) (15.5 units per ml), and 4.8 μM 1\(^{\text{3}}\)H-labelled in 50 mM sodium phosphate buffer pH 7.0. The \( \text{O}_2 \) scrubbing solution was left in the flow system overnight and was thoroughly rinsed with anaerobic buffer before experiments. The rate of \( \text{C}_2 \) mutants with \( \text{O}_2 \), the reduced enzyme in presence and absence of substrate (HPA) in 50 mM sodium phosphate buffer pH 7.0 was measured at 300 K and reported in Table S2. Rate constants were obtained by fitting a Levenberg-Marquardt nonlinear fit of \( k_{\text{obs}} \) versus \( \text{O}_2 \) concentrations, as implemented in the KaleidaGraph software (35). **SI Text** reports experimental details. **AldO.** Purified mutant and wild-type AldO were obtained as described in ref. 37. Rapid kinetics measurements were performed using an Applied Photophysics stopped-flow apparatus model SX17TM following methods reported in ref. 37. Reduced AldO was used at 3.5 μM and substrates were prepared as described in ref. 37. Reduced AldO was prepared by anaerobic buffer reduction. All experiments were performed at 300 K and in a 50 mM potassium phosphate buffer pH 7.5. Experimental details are reported in **SI Text**.

**ACKNOWLEDGMENTS.** This work was supported by the National Science Foundation Grant PHY-0822283; the National Institutes of Health and National Institute of General Medical Sciences Grant 7726 (R.B. and J.A.M.); the Howard Hughes Medical Institute (J.A.M.); the National Science Foundation Petroleum Research Fund Grant 46271-C4 and Ministeri dell’Istruzione, dell’Universita’ e della Ricerca (A.M.); the EU FP7 “Oxygen” project (A.M. and M.W.F.); the Carbohydrate Research Center Wageningen (to W.J.H.v.B.); the Thailand Research Fund grant BRG51800002 and the Faculty of Science, Mahidol University (P. Chaiyen); the Institute for the Promotion of Teaching Science and Technology (P. Chenprakhon); Royal Golden Jubilee PhD Program Grant PhD0082549 (K.T.); the Danish Technology Foundation Stichting Technische Wetenschappen Grant 7726, the Nederlandse Organisatie voor Wetenschappelijk Onderzoek applied science division, and the Technology Program of the Ministry of Economic Affairs (R.T.W.) We thank the Center for Theoretical Biological Physics for the computing resources.