Exploring Molecular Oxygen Pathways in *Hansenula polymorpha* Copper-containing Amine Oxidase*§*

Received for publication, February 14, 2007, and in revised form, March 26, 2007 Published, JBC Papers in Press, April 4, 2007, DOI 10.1074/jbc.M701308200

Bryan J. Johnson†, Jordi Cohen‡, Richard W. Welford*, Arwen R. Pearson‡, Klaus Schulten‡, Judith P. Klinman*, and Carrie M. Wilmot†*

From the †Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, Minnesota 55455, §Beckman Institute, University of Illinois, Urbana-Champaign, Illinois 61801, and ‡Departments of Chemistry and Molecular and Cellular Biology, University of California, Berkeley, California 94720

The accessibility of large substrates to buried enzymatic active sites is dependent upon the utilization of proteinaceous channels. The necessity of these channels in the case of small substrates is questionable because diffusion through the protein matrix is often assumed. Copper amine oxidases contain a buried protein-derived quinone cofactor and a mononuclear copper center that catalyze the conversion of two substrates, primary amines and molecular oxygen, to aldehydes and hydrogen peroxide, respectively. The nature of molecular oxygen migration to the active site in the enzyme from *Hansenula polymorpha* is explored using a combination of kinetic, x-ray crystallographic, and computational approaches. A crystal structure of *H. polymorpha* amine oxidase in complex with xenon gas, which serves as an experimental probe for molecular oxygen binding sites, reveals buried regions of the enzyme suitable for transient molecular oxygen occupation. Calculated O2 free energy maps using copper amine oxidase crystal structures in the absence of xenon correspond well with later experimentally observed xenon sites in these systems, and allow the visualization of O2 migration routes of differing probabilities within the protein matrix. Site-directed mutagenesis designed to block individual routes has little effect on overall $k_{cat}/K_m$ (O2), supporting multiple dynamic pathways for molecular oxygen to reach the active site.

Copper amine oxidases are ubiquitous copper containing enzymes that oxidize primary amines to aldehydes through the reduction of molecular oxygen to hydrogen peroxide. CAO catalysis is dependent upon the protein-derived cofactor 2,4,5-trihydroxyphenylalaninequinone (TPQ). The TPQ is derived from an endogenous tyrosine through a self-catalytic process requiring only molecular oxygen and Cu(II) (see Fig. 1a) (1). *Hansenula polymorpha* amine oxidase is the eukaryotic CAO that has been kinetically characterized in the most detail (2–7). HPAO follows a Bi Bi ping-pong reaction mechanism that can be expressed as two half-reactions, reductive and oxidative (see Fig. 1b). In the reductive half-reaction the enzyme oxidizes a primary amine to an aldehyde, generating the 2e− reduced aminoalcohol form of the cofactor. In the subsequent oxidative half-reaction molecular oxygen is reduced to hydrogen peroxide via cofactor reoxidation to TPQ. Biochemical studies from several different laboratories have led to mechanistic proposals for the catalytic cycle of CAOs (8, 9). These studies have given significant insight into the mechanism for the reductive half-reaction (10). However, the details surrounding the activation of molecular oxygen both in terms of the biogenesis of the TPQ (11, 12) and of the catalytic oxidative half-reaction, remain the subject of intense study (13–16). The utilization of copper as a redox center has been the focus of recent controversy. Because CAOs contain a copper ion in their active site, chemical intuition suggests Cu(I) as the O2-activating species to give Cu(II)-superoxide (17). Upon anaerobic amine reduction of CAOs, an equilibrium between Cu(II)-aminoalcohol and Cu(I)-semiquinone is observed, with Cu(I)-semiquinone yields varying from 0 to 40% depending on the enzyme source (18, 19). Plant CAOs have high yields of Cu(I)-semiquinone and bacterial CAOs are in the middle of the range, whereas non-plant eukaryotic CAOs have minimal or undetectable amounts of Cu(I) after amine reduction. It has been postulated that despite the unobservable amount of Cu(I)-semiquinone in many non-plant eukaryotic CAOs, there must still be enough Cu(I) present for this to be the O2-activating species during catalysis. However, Co(II)-substituted HPAO has a $k_{cat}$ almost identical to Cu-HPAO at pH 7 (2, 6). The reduction potential for Co(II)/Co(I) (e.g. −0.4 to −0.5 V versus SHE in methionine synthase) (22) makes Co(I) an unlikely intermediate in catalysis and effectively rules out the requirement for a copper redox change during HPAO catalysis. Addi...
Molecular Oxygen Pathways in Copper Amine Oxidase

tionally, kinetic isotope effects, steady state kinetics, viscoso-
gen, and stopped-flow experiments have shown that the re-
duction of molecular oxygen contributes 29% to the overall
\[ k_{cat} \] of 3, 23. This is a surprising result because the reduction of
O_2 by Cu(I) to give superoxide is likely to be extremely fast (17).
Based on these results a new mechanism was proposed for both
HPAO and bovine serum amine oxidase that invoked an off-
metal molecular oxygen binding pocket for the first electron
transfer to O_2 directly from the aminooquinol (2). The off-metal
binding site at which O_2 undergoes the initial one-electron
reduction was proposed to be adjacent to the metal, bounded by
the side chains of Met-634, Leu-425, and Tyr-407 (24). Site-
directed mutagenesis of residue Met-634 found a size depend-
ence on \[ k_{cat}/K_m \] in O_2 in support of this proposal.

Xenon complexation can be used to probe the interior of
protein structures for sites that favor molecular oxygen gas
binding. Because of its analogous properties in size and hydro-
phobicity, any region that binds xenon is proposed to also be
favorable for O_2 (25). In myoglobin, for instance, xenon binding
cavities observed crystallographically were also shown to bind
photolysed CO, another dioxygen mimic (26–30). X-ray crystal
structures of CAOs bound to Xe are available from bacterial
(Arthrobacter globiformis, AGAO), yeast (Pichia pastoris,
PPLO), plant (Pisum sativum, PSAO) (31), and mammalian
sources (bovine serum amine oxidase) (32). An additional solu-
tion phase NMR study found that Xe also bound to lentil seed-
ling CAO (Lens esculenta), and the data implicated a Xe-in-
duced conformational change at the active site during amine
reduction (33). In the four available crystal structures there is
one consistent xenon site that is ~7 Å from the copper and
~7.5 Å from the TPQ. We term this site the “anteroom,” and
the residues bordering this pocket in HPAO are Leu-425, Tyr-
407, Phe-460, Ile-423, Ile-622, and Ile-639 (see Fig. 2). The ante-
room is too far from either the copper or aminooquinol to be
directly involved in the reduction of molecular oxygen to super-
oxide but could act as a holding pocket for O_2 close to the active
site, and is consistent with results that indicate O_2 is pre-bound
to the enzyme before reduction (3, 24). The off-metal hydro-
phobic pocket proposed through mutagenesis studies at Met-
634 in HPAO is adjacent to the anteroom, and the two pockets
share a common residue, Leu-425 (see Fig. 2) (2). However, a
L425A mutant had surprisingly little effect on \[ k_{cat}/K_m \] of O_2 (2).

The shortest distance from the anteroom to the protein-sol-
vent boundary in CAOs (~10 Å long) follows a chain of ordered
waters from the equatorial water ligand of the copper ion to an
area of solvent termed the “inland lake” (see Fig. 2) (34, 35). The
inland lake is conserved at the dimer interface of CAOs and has
been suggested as the entry point for O_2 (35). However, as a
polar channel often only one water molecule wide, it is hard to
reconcile this with the hydrophobic nature of molecular oxy-
gen. To help resolve all these data, we wanted to delineate more
clearly molecular oxygen holding pockets in HPAO as well as
define other potential molecular oxygen pathways into the bur-
ied active site.

Here we report a crystal structure of HPAO in complex with
xenon that offers new insights into CAO-molecular oxygen
interactions. Bound xenon in the dense hydrophobic interior of
the catalytic domain suggests a putative channel within CAOs
accessibile to molecular oxygen. Free energy maps of molecular
oxygen, computed from dynamics simulations of several CAOs
in the absence of xenon, correlate with experimental observa-
tions and suggest new pathways that molecular oxygen might
take to the buried active site.

EXPERIMENTAL PROCEDURES

HPAO Purification—Wild-type HPAO (wHPAO) for crys-
tallization was heterologously expressed in Saccharomyces cere-
visiae and purified as described previously (7, 36) with modi-
fications. In brief, single colonies from URA^- plates incubated
at 30 °C were streaked and used to inoculate URA^- liquid
medium supplemented with 10 μM CuSO_4 and a sterile filtered
nucleotide/amino acid mixture (5.0 g/liter adenine, 5.0 g/liter
histidine, 7.5 g/liter leucine, and 5.0 g/liter tryptophan).

The preparation of the URA^- media contained 1.7 g/liter of yeast
nitrogen base (without amino acids) to minimize the Zn^{2+} con-
centration in the media. Previous protocols called for 6.7 g/liter of
yeast nitrogen base (36). Once they had reached an A_600 of
5 ~6, the cells were collected by centrifugation and ruptured by
Bead-Beater (Biospec). The soluble fraction of the freshly lysed
cells was not dialyzed as previously reported but was, instead,
equilibrated into 5 mM potassium phosphate buffer by 10-fold
dilution. The diluted soluble fraction was then immediately
loaded onto the DEAE anion exchange column. This was fol-
lowed by size exclusion chromatography on a Sephadex S-300
column. The protein was buffer exchanged into 50 mM HEPES,

pH 7.0, and concentrated to 13 mg/ml before crystallization.

HPAO Crystallization—Crystallization was performed as
previously reported (13 mg/ml HPAO in 8.0–9.5% polyethy-
lene glycol 8000, 0.28–0.30 M phosphate at pH 6.0 at 293 K) with
the exception of the use of hanging drop crystallization rather
than sitting drop (34). The ratio of protein to well solution in
the hanging drops was 1:1, with volumes of 2.5 μl, giving a total
volume of 5.0 μl. The best diffracting crystals were cube-shaped
with dimensions of 40 × 40 × 30 μm and grew within 3 days.
The space group of the crystals used in this study was P2_1,
although crystals also grew in the space groups C2 and P2_1,2_1
from the same crystallization conditions. Cryoprotection was
performed by soaking crystals for 10 s in 25% high purity glyc-
erol (Hampton Research) mixed with mother liquor directly
from the crystallization well.

Xenon Complexation—Crystals of HPAO in cryoprotected
mater liquor were complexed with xenon using a pressure cell
(Rigaku) at a pressure of 10 atm for 10 min. To prevent dehy-
drination of the crystal during the pressurization, a small knot
of water-saturated Kimwipe was placed at the bottom of the
chamber near where the loop containing the crystal would rest
during the pressurization. Before pressurization the crystals
were soaked in cryoprotectant. Within 5–10 s of depressuriza-
tion the crystals were flash-frozen in liquid nitrogen.

Single Crystal Microspectrophotometry—Single crystal spec-
tra were collected at 100 K using the 4DX Systems AB
microspectrophotometer equipped with an MS125™ 1/8 m
spectrograph (Thermo Oriel), CCD detector (Andor Tech-
ology), and xenon lamp (Zeiss) emitting from 300 to 800
nm. Spectra were generated from the integration of 10 19-ms
exposures (37).
Molecular Oxygen Pathways in Copper Amine Oxidase

Structure Determination and Refinement—Each x-ray data set was collected from a single crystal at 100 K at the Advanced Photon Source, Argonne National Laboratory (Beamline ID-19, SBC-CAT). High resolution diffraction data were measured at λ = 0.979 Å (Table 1). For the xenon complex, data were collected at two detector distances; one for the optimal collection of high resolution data and another for the minimization of overlaps at lower resolution. These two data sets were merged for optimal completeness. From the same crystal, an additional lower resolution data set was collected at λ = 1.72 Å to optimally collect anomalous scattering associated with the bound xenon (for Xe f ′′ = 9.0 electrons) (supplemental Table S1). Data were processed with HKL2000 and SCALePACK (38). Molecular replacement was performed with MOLREP, part of the CCP4 suite (39), using the previously deposited HPAO model. Protein Data Bank (PDB) code 1a2v. Calculation of the anomalous map was performed using the programs of the CCP4 suite (39). Model building was performed using COOT (40). Refinement of the model was performed using REFMAC5 (41). There was no use of non-crystallographic symmetry restraints during refinement. Waters were placed in the model at peaks >3.0 σ in the 2Fo − Fc map using ARP waters, part of REFMAC5 (39). Atomic coordinates for the position of xenon atoms were located by 6-fold averaging of the anomalous map. Peaks above 6.5 σ were assigned to xenon in the xenon complex provided that they did not overlap with other anomalous scatterers at this wavelength, including copper and sulfur, and were in agreement with expected hydrophobic backbone and side-chain interactions. Individual Xe occupancies were set at the occupancy that equated Xe B-factors to those of the surrounding protein during refinement and absence of Fo − Fc electron density.

Mutagenesis—Mutations were made to the pDB20-HPAO plasmid (7) using the Stratagene QuikChange kit. Primers were purchased high performance liquid chromatography-purified from Operon. The forward primers are given below; the reverse primers were complementary to these. The mutated codon is given in bold, and the changed bases are underlined. Sequences were confirmed by automated DNA sequencing (University or California, Berkeley, CA). The mutated plasmids were transformed into the S. cerevisiae cell line CG379 (ATCC) by lithium acetate chemical transformation. Sequences are: L425F, 5′-CACAGCTCGACATCGAGATTCA-CGGTGATTCTGCAC-3′; I622Y, 5′-CTTCCATACTTTC-CCTACAAATTTTACCGCT-3′; L643F, 5′-CCCTATCACCCTGTGTAGTTAAGACTTCCGCAC-3′; I639F, 5′-GATGCGCTGCCAGCTTTCACCTTTGATGCTTAG-3′.

Mutant Characterization—TPQ content was determined by titration against phenylhydrazine in 100 mM KPi, pH 7.2, at 30 °C and measuring the change in absorbance at λ = 448 nm using ε = 40,500 M−1 cm−1. TPQ per subunit concentrations were then calculated using a total protein concentration kept constant at 0.3M by the addition of an appropriate amount of KCl. The oxygen concentration was kept constant at 25 °C; reactions initiated by the addition of HPAO. For determinations of kcat/Km (O2), the methyleneamine concentration was kept constant at 5 mM, whereas for determinations of kcat/Km (methylamine), the oxygen concentration was kept constant at 258 μM. Solutions were equilibrated to atmospheric conditions by stirring at 1000 rpm for 5 min just before initiation. For reactions at different oxygen concentrations, two flow meters were used to regulate the flow of O2 and N2, and the equilibration time was extended to 10 min. For assays in the pH range 6–8 and 8–9, 100 mM KPi and 25 mM pyrophosphate buffers were used, respectively. The ionic strength of all buffers was kept constant at 0.3 M by the addition of an appropriate amount of KCl. Data were fit directly to the Michaelis-Menten equation, and kcat was calculated using the active protein concentration as determined by phenylhydrazine titration. The variation of kcat/Km (O2) with pH best fit to an increase with 2 pKa model using the equation,

\[
\log(x) = \log(x)_{\text{max}} - \log(1 + 10^{pK_{a} - pH} + 10^{pK_{a} + pK_{b} - 2pH})
\]

where x = kcat/Km (O2).

O2 Free Energy Maps—Computational analysis was used to predict the location of preferred pathways taken by O2 to reach the active sites of HPAO, AGAO, PPLO, and PSAO. The starting co-ordinates for each simulation were the native oxidized structures and, thus, contained no information regarding experimentally determined Xe binding sites. The protein PDB coordinates (HPAO, 2oox; AGAO, 1w6g; PPLO, 1w7c; PSAO, 1ksi) were first solvated in a 50 mM NaCl water box. If the TPQ was in the inactive “copper-on” conformation (HPAO and PPLO), it was modeled into the active “copper-off” conformation before molecular dynamics. Keeping everything else fixed, the solution was then equilibrated for 30 ps followed by a combination of the solution and protein side chains for 50 ps and, finally, the entire system for 950 ps using the NAMD simulation program (42). Trajectories were then recorded for 10-ns simulations performed at constant pressure (1 atm, Langevin piston) and temperature (300 K, Langevin dynamics) using long-range PME electrostatics and the CHARMM22 force-field (43) with custom parameters built by analogy for the TPQ residue and histidine-copper bonds. The trajectories were used as input for an implicit ligand sampling analysis (44) contained in the VMD software package (45), resulting in detailed three-dimensional free energy profiles for O2 placement inside the protein based on the assumption that the presence of gas molecules can be
Molecular Oxygen Pathways in Copper Amine Oxidase

TABLE 1
Data collection and refinement statistics

|                      | Native HPAO | Xe-complexed HPAO |
|----------------------|-------------|-------------------|
| Data collection      |             |                   |
| Detector type        | ADSC-CCD    | ADSC-CCD          |
| Source               | APS         | APS               |
| Space group          | P2₁         | P2₁               |
| Unit cell (Å)        | 104.1 x 223.1 x 104.3 | 103.4 x 222.8 x 103.7 |
| Wavelength (Å)       | 0.9785      | 0.9785            |
| Resolution (Å)       | 50-1.70 (1.74-1.70) | 50-1.60 (1.64-1.60) |
| Measured reflections | 2,007,440   | 1,612,831         |
| Unique reflections   | 437,335     | 587,360           |
| Completeness (%)     | 85.0 (32.1) | 96.3 (72.9)       |
| R_{min} (%)          | 0.085 (0.550) | 0.079 (0.373)    |
| I/σ(F)               | 14.5 (1.4)  | 21.4 (1.4)        |
| Redundancy           | 4.6 (2.8)   | 2.7 (1.6)         |
| Refinement           |             |                   |
| Resolution (Å)       | 38.0-1.70 (1.74-1.70) | 47.0-1.60 (1.64-1.60) |
| Number of reflections; working set/test set | 437,276/21,965 (12,226/606) | 587,333/29,665 (29,592/1,585) |
| R-factor             | 14.6 (22.6) | 16.3 (27.8)       |
| R-free               | 17.7 (27.6) | 18.9 (31.9)       |
| Protein atoms        | 31,432      | 31,594            |
| Water molecules      | 5,054       | 3,803             |
| Other atoms          | 177         | 293               |
| Root mean square deviation | 0.011 | 0.010 |
| Bond lengths (Å)     | 1.33        | 1.34              |
| Bond angles (°)      | 22.7        | 20.2              |
| Average B-factor (Å²) | 0.093      | 0.076             |
| DPI (Å²)             |             |                   |

Numbers in parentheses represent values in the highest resolution shell.

The data had excellent completeness to 1.91 Å resolution, where overall completeness was 94.2%, and the data were 94.2% complete in the 2.02-1.91-Å resolution shell.

However, there are 6 monomers in the asymmetric unit, 2 dimers have mixed TPQ orientations composed of both active copper-off and inactive copper-on (TPQ axially ligated to copper via O4) conformations and were modeled as such. The copper-off active sites also show the presence of an oxidized methionine (Met-634) near the Cu(II) ion, with no evidence of oxidation at any other methionines in the structure. This site is probably particularly sensitive to radiation damage due to its proximity to the redox active site of the enzyme. It is difficult to judge whether Met-634 is oxidized in the subunits with both copper-on and copper-off TPQ conformations because the copper-on form obscures the electron density of that position.
To further investigate the relevance of xenon binding pockets in CAOs in influencing reactivity with molecular oxygen, we designed several point mutations at residues around these sites (Table 2). The residue Leu-425, which divides the two proposed O2 binding pockets (Fig. 2), was mutated to phenylalanine to investigate the effects of increasing residue size and hydrophobicity at this position. Ile-622 is a possible candidate residue for controlling entry of gaseous molecules into the anteroom from the inland lake, and so this was mutated to a tyrosine in an effort to block entry to this site (Fig. 2). The side chain of Ile-639 sits in the anteroom and is comparatively remote from the copper, so this residue was mutated to a phenylalanine in an attempt to restrict gas binding (Fig. 2). Finally a L643F mutation was made at the Xe1 site in the hydrophobic core of the catalytic domain in an attempt to disrupt gas conduction through this region (Fig. 3b).

All four mutants had 1 copper and 0.5 TPQs per subunit as for wtHPAO, except I622Y, which had 1 copper and 0.1 TPQ per subunit (supplemental Table S2). The Cu(II) EPR spectra of L425F, I639F, and I622Y were all similar to wtHPAO, indicating that the primary coordination environment of the copper had not been perturbed (supplemental Table S3). The limiting rate constant, \( k_{\text{cat}} \), for all the mutants was reasonably similar to that of wt, with the slowest being down not more than 2-fold. The value of \( k_{\text{cat}}/K_m \) (methylamine) was similar to that of wtHPAO for all four mutants, indicating little change in the reductive half-reaction. This is to be expected as these mutations are remote from the site where the chemistry for this step occurs. When \( k_{\text{cat}}/K_m \) was measured at pH 8, I639F, I622Y, and L643F gave very similar values to wtHPAO, but that for L425F was down by an order of magnitude (Table 2). To see if the I639F and L425F mutations affected the active site or the anteroom, \( k_{\text{cat}}/K_m \) was measured as a function of pH (supplemental Fig. S2). Previously, the variation of \( k_{\text{cat}}/K_m \) with pH for wtHPAO was found to fit a 2 pK\(_a\) model, with both deprotonations increasing the rate (3). The pK\(_a\) values of 6.8 ± 0.1 and 7.9 ± 0.1 have been assigned to the protonated form of the aminoquinol cofactor and the Cu-OH\(_2\), respectively. For both L425F and I639F, the data fit well to a 2 pK\(_a\) model. For I639F the pK\(_a\) values (6.8 ± 0.3 and 7.7 ± 0.2) were essentially the same as wtHPAO, whereas for L425F they were both up by 0.5 pH units (7.3 ± 0.2 and 8.4 ± 0.1). The altered pK\(_a\) values for L425F indicate that this mutation is directly influencing the active site while that of I639F is not.

The migration of molecular oxygen through CAOs from a protein-wide perspective was explored using a computational method called implicit ligand sampling (44), developed for finding gas migration pathways inside proteins. This method takes advantage of the fact that O\(_2\) travels along transient cavities in the protein that continuously form and disappear over relatively short timescales (1 ns). By monitoring these transient cavities as they appear over a 10-ns molecular dynamics window in the protein, even in the absence of O\(_2\), the potential of mean force (PMF) corresponding to the placement of O\(_2\) everywhere inside the protein can be computed (44). The result is a complete three-dimensional map of the free energy of placing an O\(_2\) molecule anywhere inside a protein. Networks of potential O\(_2\) entry and exit pathways inside the protein are inferred by connecting favorable areas for O\(_2\) that are in close proximity to one another forming gas migration pathways. Because the thermal fluctuations responsible for the migration of gas molecules such as O\(_2\) inside proteins are well sampled during the simulation time used (10 ns), the computed O\(_2\) pathways are highly probable (with the caveat that possible O\(_2\) pathways which are caused by rare events may not be identified by this method).

This approach has been applied here to HPAO, AGAO, PPLO, and PSAO to correlate the experimentally observed xenon sites with calculated pathways (Fig. 4 and supplemental data files pmf-xxxx-02-10ns.dx (O\(_2\) free energy maps) and rotated-xxxx-equilibrated.pdb (rotated co-ordinates to match maps), where xxxx = hpaO, agao, pplo, psao). Although the starting models in each case had TPQ modeled in the active copper-off conformation, where TPQ hydrogen bonds via its O2 position to the axial water ligand of the copper (Fig. 4c), in AGAO and PPLO the TPQ migrated onto the copper during the 1-ns equilibration that is part of the free energy calculation (Fig. 4, d and e). This copper-on form, in which the TPQ displaces the axial water ligand to directly ligate to the copper via its O4 position, is a common conformation observed in CAO crystal structures (including the HPAO structures presented here) and is required for the biogenesis of TPQ (35, 48). None of the calculated pathways were affected by this change in TPQ
**Molecular Oxygen Pathways in Copper Amine Oxidase**

![Image](https://example.com/image.png)

**FIGURE 3.** Xenon binding sites in HPAO. **a,** overall view of the HPAO dimer. One monomer is displayed and colored by domain: **blue,** D1; **yellow,** D2; **green,** D3. The monomer displayed in Ca trace has the TPQ in stick, and the copper as a green sphere. Xenons are displayed as magenta spheres, and the numbering corresponds to peak size in the anomalous map. **b,** Xe1 within the D3 β-sandwich. **c,** Xe2 and Xe3 within the substrate amine channel. The **black mesh** represents the anomalous map contoured at 9.5 σ. Residues with side chains within van der Waals contact are displayed. Residues in **gray** belong to the HPAO + Xe complex, and those in **blue** belong to oxidized HPAO. The figure was generated using Pymol (20).

Conformation. The location of experimental Xe binding correlates well with the locations of minimum free energy computed from implicit ligand sampling on a 10-ns molecular dynamics simulation in each CAO (supplemental Fig. S3). The only exceptions are surface-bound Xe sites (Xe4 in HPAO and Xe903 in AGAO). In addition, implicit ligand sampling provides a complete three-dimensional map for the migration of O₂ between favorable regions. This map is created by contouring energy isosurfaces representing elevated PMF values (1.8 and 3.0 kcal/mol, Fig. 4). In HPAO, we identify two major regions that contain the most probable pathways from protein solvent boundary to the buried active site according to the implicit ligand sampling calculation (Fig. 4b). One starts from the amine entry channel close to the 5 position of the TPQ cofactor where the reductive half-reaction chemistry is known to take place (left pathway). Another leads through the hydrophobic core of the β-sandwich toward the active site (right pathway). The two pathways merge near the active site, and there is an energetically favorable O₂ docking site that could play a role in pre-binding O₂ in HPAO before it enters the active site for activation (Fig. 4c). This is distinct from the previously described anteroom and is consistent with the lack of an anteroom Xe binding site in HPAO. The PMF maps of AGAO, PPLO, and PSAO have a larger (less favorable) PMF value in the area of the HPAO docking site, and in these enzymes the anteroom is the most favorable area close to the active site for pre-binding O₂. The AGAO PMF maps suggest that the most favorable migration route is from the D3 β-sandwich hydrophobic core through the anteroom to the active site, which is similar to that suggested by the HPAO PMF maps (Fig. 4d). In PPLO and PSAO, passage via this route appears much less favorable (Fig. 4, e and f). In all cases the short polar channel that exists between the inland lake and the active site, which had previously been proposed as the probable entry site for molecular oxygen, is not the only pathway between these two areas (35). Rather, there are connections from the inland lake to the anteroom, particularly in PPLO and PSAO (Fig. 4, e and f) where an equally favorable route appears to occur near, but distinct from the previously described polar channel.

**DISCUSSION**

The location of Xe1 in the HPAO + Xe complex has revealed the presence of a binding region that is not accessed by a traditionally defined channel. Xe1 represents ligand binding in a solvent-inaccessible void. The puzzling nature of this singular site in HPAO provoked us to look for similar sites in other CAOs. In examining a composite overlay of all available xenon complex data, totaling four different species, we were immediately struck by the chain of Xe atoms that occupied the hydrophobic core of the catalytic β-sandwich (Fig. 5). The overlaid sites are not coincident, yet they appear to mark a highly favorable area for a small hydrophobic molecule, such as molecular oxygen, to reside. This chain of Xe atoms also appears to indicate a pathway whereby molecular oxygen might enter at either end of the catalytic β-sandwich and then proceed toward the active site, passing through or close by the anteroom along the way (arrows, Fig. 5b). Indeed, the lining of the catalytic β-sandwich with larger hydrophobic residues well suits it for the favorable accommodation and conduction of molecular oxygen (Fig. 3b). Large hydrophobic side chains, like those found in this region, are highly flexible and mobile, offering a higher propensity for packing defects that allows the passage of small hydrophobic molecules. The sequence conservation in this region for the CAOs is similar to that seen for any large protein hydrophobic core, suggesting there is nothing spe-
cial about the CAO catalytic β-sandwich in terms of O₂ migration.

An additional striking feature of the CAO + Xe composite overlay was the presence of Xe atoms in the amine channel. In the HPAO + Xe complex the amine channel supports the binding of two atoms of xenon in place of ordered waters normally found in the oxidized structure (Fig. 3c and 5a, upper inset). AGAO provides an additional site nearer the surface of the enzyme. As part of the amine channel, these observed sites are on the opposite side of the cofactor from the location of dioxygen chemistry (49). In addition to marking a region suitable for O₂ binding, the closest peak to the TPQ in HPAO, Xe3, probably indicates a favorable binding site for the aliphatic portion of the small amine substrates favored by this CAO and helps orient the substrate for correct nucleophilic attack at the C5 of TPQ. In HPAO, orientation of substrate would be mediated through a favorable hydrophobic interaction with Trp-156 (Fig. 3c). By structural overlay, the position of Trp-156 aligns it with gating residues found in other CAOs (35). CAOs generally have a tyrosine or a phenylalanine at this position that is mobile and acts like a “gate” between the amine channel and the TPQ (32, 35, 50–55). In the case of ECAO, Tyr-381 is known to stack on

**FIGURE 4.** Implicit ligand sampling performed in CAOs with a molecular oxygen probe. a, a ribbon representation of HPAO that is indicative of the orientation and slice depth of the subsequent PMF maps. Shown are PMF maps of HPAO (b), AGAO (d), PPL0 (e), and PSAO (f). Surfaces: red opaque represents a 0 kcal/mol isosurface; binding regions for molecular oxygen. Blue transparent represents a 1.8 kcal/mol isosurface; regions of molecular oxygen occupancy more favorable than occupation in solvent. Black mesh represents a 3.0 kcal/mol isosurface; lowest energy pathways between regions of binding. The gray/black surface represents the monomer surfaces. Most favorable routes for O₂ from the surface are marked by green lines. Residues shown include the TPQ and histidine ligands of the Cu(II) (shown as a green sphere). The box in the HPAO map (b) is enlarged (c) to show a close-up of the HPAO active site with PMF map displayed. Surrounding residues are colored in purple and green, corresponding to the energetically favorable HPAO O₂ binding site and the anteroom, respectively. a and c were generated using Pymol (20). b, d, e, and f were generated using VMD (45).
Molecular Oxygen Pathways in Copper Amine Oxidase

![Image](50x460 to 407x734)

**FIGURE 5. Comparison of CAO xenon binding sites.** a, overlay of CAO xenon sites deposited in the PDB with a monomer of the HPAO/Xe complex. The backbone of HPAO is displayed and colored by domain: blue stick, D1; yellow stick, D2; green ribbon, D3. Xenon sites from individual complexes are coded by color (red, PSAO (PDB code 1rjo); blue, AGAO (PDB code 1rky); yellow, PPLO (PDB code 1rko); magenta, HPAO (PDB code 2ooq)), except surface-bound xenon sites, which are displayed as gray spheres (31). The top inset shows the amine channel. The bottom inset shows a proposed molecular oxygen pathway identified in this study. The active site is shown in stick, and the copper is shown as a green sphere. b, the HPAO D3 β-sandwich domain viewed from the dimer interface (90° rotation, cf. view in a). Only the xenon sites in the internal D3 β-sandwich and close to the anteroom are shown. Colors are the same as in a, and arrows indicate the direction of molecular oxygen movement to the active site using the proposed pathway. The figure was generated using Pymol (20).

The analysis of putative molecular oxygen pathways was taken one step further by the generation of PMF maps through implicit ligand sampling analysis. PMF maps reveal two important features present in protein structures. The first feature is the location of hydrophobic binding sites in CAOs that are favorable for small hydrophobic molecules and which may or may not correspond to preexisting static cavities. This is accomplished by generating surfaces at the 0 kcal/mol energy level, which correspond to areas of the protein where the free energy of placing O₂ is more favorable than in a vacuum of the same volume. The second feature is the lowest energy route available between determined binding sites by generating iso-surfaces at elevated PMF levels. The free energy map calculation in HPAO confirms with excellent fidelity the experimentally observed xenon binding sites across CAOs within the core of the catalytic β-sandwich (supplemental Fig. S3). By elucidating several regions within the catalytic β-sandwich (D3 domain) that are favorable and accessible to molecular oxygen binding, we can visualize a series of binding sites potentially used en route to the active site (Fig. 4b). Similar binding possibilities in the β-sandwich are seen in map calculations for AGAO, PPLO, and PSAO, with a route being most apparent in AGAO (Fig. 4d). The molecular oxygen pathways vary slightly between CAOs, especially as they near the active site, and suggest that use of the anteroom is not an absolute requirement. However in PPLO, PSAO, and AGAO, where utilization of the anteroom has been suggested by Xe complexation, the free energy maps suggest a high probability for O₂ binding in that region (31).

The HPAO PMF maps revealed an equally energetically favored O₂ pathway to the buried active site through the amine channel. In the case of a partially polar molecule, such as the amine, this pathway terminates near the O5 position of the cofactor. However, when considering O₂, PMF maps show that the path instead continues past the backside of the TPQ and merges with the β-sandwich route near an HPAO-specific binding site (Fig. 4c). Due probably to the HPAO narrow amine channel structure and the presence of some hydrophobic residues, this channel is equally suited to conduct O₂ as well as the aromatic portion of its preferred aromatic monoamine substrates and has a stabilizing and orienting effect (54). Because no side-chain movement is involved in the binding of Xe2 in HPAO, Trp-156 does not seem to act as a gate per se, being already in the open conformation (Fig. 3c).

The analysis of putative molecular oxygen pathways was taken one step further by the generation of PMF maps through implicit ligand sampling analysis. PMF maps reveal two important features present in protein structures. The first feature is the location of hydrophobic binding sites in CAOs that are favorable for small hydrophobic molecules and which may or may not correspond to preexisting static cavities. This is accomplished by generating surfaces at the 0 kcal/mol energy level, which correspond to areas of the protein where the free energy of placing O₂ is more favorable than in a vacuum of the same volume. The second feature is the lowest energy route available between determined binding sites by generating iso-surfaces at elevated PMF levels. The free energy map calculation in HPAO confirms with excellent fidelity the experimentally observed xenon binding sites across CAOs within the core of the catalytic β-sandwich (supplemental Fig. S3). By elucidating several regions within the catalytic β-sandwich (D3 domain) that are favorable and accessible to molecular oxygen binding, we can visualize a series of binding sites potentially used en route to the active site (Fig. 4b). Similar binding possibilities in the β-sandwich are seen in map calculations for AGAO, PPLO, and PSAO, with a route being most apparent in AGAO (Fig. 4d). The molecular oxygen pathways vary slightly between CAOs, especially as they near the active site, and suggest that use of the anteroom is not an absolute requirement. However in PPLO, PSAO, and AGAO, where utilization of the anteroom has been suggested by Xe complexation, the free energy maps suggest a high probability for O₂ binding in that region (31).

The HPAO PMF maps revealed an equally energetically favored O₂ pathway to the buried active site through the amine channel. In the case of a partially polar molecule, such as the amine, this pathway terminates near the O5 position of the cofactor. However, when considering O₂, PMF maps show that the path instead continues past the backside of the TPQ and merges with the β-sandwich route near an HPAO-specific binding site (Fig. 4c). Due probably to the HPAO narrow amine channel structure and the presence of some hydrophobic residues, this channel is equally suited to conduct O₂ as well as the aromatic portion of its preferred aromatic monoamine substrates and has a stabilizing and orienting effect (54). Because no side-chain movement is involved in the binding of Xe2 in HPAO, Trp-156 does not seem to act as a gate per se, being already in the open conformation (Fig. 3c).

The analysis of putative molecular oxygen pathways was taken one step further by the generation of PMF maps through implicit ligand sampling analysis. PMF maps reveal two important features present in protein structures. The first feature is the location of hydrophobic binding sites in CAOs that are favorable for small hydrophobic molecules and which may or may not correspond to preexisting static cavities. This is accomplished by generating surfaces at the 0 kcal/mol energy level, which correspond to areas of the protein where the free energy of placing O₂ is more favorable than in a vacuum of the same volume. The second feature is the lowest energy route available between determined binding sites by generating iso-surfaces at elevated PMF levels. The free energy map calculation in HPAO confirms with excellent fidelity the experimentally observed xenon binding sites across CAOs within the core of the catalytic β-sandwich (supplemental Fig. S3). By elucidating several regions within the catalytic β-sandwich (D3 domain) that are favorable and accessible to molecular oxygen binding, we can visualize a series of binding sites potentially used en route to the active site (Fig. 4b). Similar binding possibilities in the β-sandwich are seen in map calculations for AGAO, PPLO, and PSAO, with a route being most apparent in AGAO (Fig. 4d). The molecular oxygen pathways vary slightly between CAOs, especially as they near the active site, and suggest that use of the anteroom is not an absolute requirement. However in PPLO, PSAO, and AGAO, where utilization of the anteroom has been suggested by Xe complexation, the free energy maps suggest a high probability for O₂ binding in that region (31).

The HPAO PMF maps revealed an equally energetically favored O₂ pathway to the buried active site through the amine channel. In the case of a partially polar molecule, such as the amine, this pathway terminates near the O5 position of the cofactor. However, when considering O₂, PMF maps show that the path instead continues past the backside of the TPQ and merges with the β-sandwich route near an HPAO-specific binding site (Fig. 4c). Due probably to the HPAO narrow amine channel structure and the presence of some hydrophobic residues, this channel is equally suited to conduct O₂ as well as
Molecular Oxygen Pathways in Copper Amine Oxidase

ligand sampling analysis, although other connections from the inland lake are also evident (Fig. 4, e and f). In the case of HPAO, however, this path is energetically unfavorable for O₂ (Fig. 4b, purple). As noted by Duff et al. (31), if molecular oxygen accesses the anteroom from the polar channel linking the inland lake to the active site, it would need to first pass through the active site and by the probable site of O₂ activation. This route seems at odds with one meant to deliver O₂ to a site of active site chemistry. Further studies are required to assess how important the favorable site, including in HPAO via the substrate amine channel. Furthermore, because there appear to be multiple routes in any given CAO, it is unlikely that a single site-directed mutant is going to impact molecular oxygen access, and this fits with the data in this study. Molecular oxygen pathways, unlike many other substrate channels, seem to rely on a delicate balance of tertiary structure, side-chain hydrophobicity, and conformational flexibility to allow the efficient transport of this small hydrophobic molecule.

In this study site-directed mutagenesis of anteroom residues I639F and I622F in HPAO that were designed to minimize or eliminate space for the binding or passage of molecular oxygen through the anteroom, lacked any difference in k_cat/K_m (O₂) values. Taken together, the I639F and I622Y mutations, the PMF maps, and the lack of xenon in this region imply that the anteroom is not a significant region for O₂ pre-binding in HPAO. This is different from AGAO, PSAO, and PPLO, where xenon complex data and implicit ligand sampling support a role for the anteroom (31). The L643F mutant of this study, which was designed to alter the side-chain size and flexibility within the hydrophobic core of the catalytic ß-sandwich at the HPAO Xe1 site, also had no effect on O₂ kinetics. This is consistent with the probability that there are multiple paths to the active site, including in HPAO via the substrate amine channel. Further studies are required to assess how important the favorable area within the D3 ß-sandwich is for O₂ channeling in HPAO, but our results indicate that single or even double point mutants may do little to significantly affect oxygen kinetics. The L425F mutant, whose bulk may affect the ability of O₂ to migrate to the site of activation, is the only one in this study to have a significant impact on k_cat/K_m (O₂). However, in light of the fact that this residue raises key pK_a values within the active site by ~0.5 pH units, its effect may be complex in terms of the active site chemistry.

Kinetic data support O₂ being pre-bound to the enzyme before activation (3, 24), which could involve one or more favorable holding areas for molecular oxygen distinct from the site of O₂ activation. We propose that the catalytic ß-sandwich and amine channel, in addition to the inland lake, serve as reservoirs of molecular oxygen, containing multiple pre-binding sites en route to the active site. In this way, multiple regions of the protein act as internal reservoirs for small hydrophobic gases, continuously supplying the active site with molecular oxygen. The apparent flexibility in approach routes for molecular oxygen in the different CAOs suggests that initial entry may not be at a single location. Instead, it is better viewed as pathways of differing probabilities where O₂ is transiently held before movement to the pocket containing Met-634, which still remains the best candidate site for off-copper activation.

The accumulated evidence from crystallography, computational, and biochemical studies on several different protein systems supports the notion that there are specific pathways for gas migration through the protein matrix (25, 59–62). The combination of molecular oxygen pathways in copper amine oxidase and molecular oxygen migration appears to follow preferred routes. There is some conservation in the pathways for molecular oxygen migration in CAOs, although their importance varies from one species to another. In particular, the hydrophobic core of the D3 catalytic ß-sandwich can act as a reservoir for molecular oxygen, with O₂ then moving into the vicinity of the active site for eventual activation. The presence of routes is largely dictated by the overall architecture of the protein, where certain secondary and tertiary structural folds, such as the hydrophobic core of the ß-sandwich found in CAOs, can create favorable reservoirs for small hydrophobic molecules such as molecular oxygen. Although the overall fold is conserved in the catalytic ß-sandwich, differences in the primary amino acid sequence probably account for the species-specific differences in the pathways. Implicit ligand sampling analysis, by monitoring transient level of cavity formation, allows the accurate prediction of hydrophobic binding regions that match with high fidelity the experimentally observed xenon binding sites. Because there appear to be multiple routes in any given CAO, it is unlikely that a single site-directed mutant is going to impact molecular oxygen access, and this fits with the data in this study. Molecular oxygen pathways, unlike many other substrate channels, seem to rely on a delicate balance of tertiary structure, side-chain hydrophobicity, and conformational flexibility to allow the efficient transport of this small hydrophobic molecule.

Acknowledgments—We thank Teresa De La Mora-Rey for help during x-ray data collection as well as the staff of Structural Biology Consortium Collaborative Access Team, especially Steve Ginell. We also thank Patton Fast of the University of Minnesota Supercomputing Institute for computer support. Use of the Advanced Photon Source was supported by the United States Dept. of Energy, Basic Energy Sciences, Office of Science, under contract W-31-109-Eng-38. In-house x-ray data collection was supported by a Minnesota Partnership for Biotechnology and Medical Genomics Grant SPAP-05-0013-P-FY06. Computer resources were provided by the Basic Sciences Computing Laboratory of the University of Minnesota Supercomputing Institute.

REFERENCES

1. Janes, S. M., Mu, D., Wemmer, D., Smith, A. J., Kaur, S., Maltby, D., Burlinge, A. L., and Klinman, J. P. (1990) Science 248, 981–987
2. Goto, Y., and Klinman, J. P. (2002) Biochemistry 41, 13637–13643
3. Mills, S. A., Goto, Y., Su, Q., Plastino, J., and Klinman, J. P. (2002) Biochemistry 41, 10517–10524
4. Plastino, J., Green, E. L., Sanders-Loehr, J., and Klinman, J. P. (1999) Biochemistry 38, 8204–8216
5. Schwartz, B., Olgin, A. K., and Klinman, J. P. (2001) Biochemistry 40, 2954–2963
6. Mills, S. A., and Klinman, J. P. (2000) J. Am. Chem. Soc. 122, 9897–9904
7. Cai, D., and Klinman, J. P. (1994) Biochemistry 33, 7647–7653
8. Mure, M., Mills, S. A., and Klinman, J. P. (2002) Biochemistry 41, 9269–9278
9. Dooley, D. M. (1999) J. Biol. Inorg. Chem. 4, 1–11
10. Klinman, J. P. (1996) Chem. Rev. 96, 2541–2562
11. Okajima, T., Kishishita, S., Chiu, Y. C., Murakawa, T., Kim, M., Yamaguchi, H., Hirota, S., Kuroda, S., and Tanizawa, K. (2005) Biochemistry 44, 10977–10984
