Reduction of dioxygen to water is a key process in aerobic life, but atomic details of this reaction have been elusive because of difficulties in observing active oxygen intermediates by crystallography. Cytochrome cd$_1$ is a bifunctional enzyme, capable of catalyzing the one-electron reduction of nitrite to nitric oxide, and the four-electron reduction of dioxygen to water. The latter is a cytochrome oxidase reaction. Here we describe the structure of an active dioxygen species in the enzyme captured by cryo-trapping. The productive binding mode of dioxygen in the active site is very similar to that of nitrite and suggests that the catalytic mechanisms of oxygen reduction and nitrite reduction are closely related. This finding has implications to the understanding of the evolution of oxygen-reducing enzymes. Comparison of the dioxygen complex to complexes of cytochrome cd$_1$ with stable diatomic ligands shows that nitric oxide and cyanide bind in a similar bent conformation to the iron as dioxygen whereas carbon monoxide forms a linear complex. The significance of these differences is discussed.

The four-electron reduction of dioxygen to water is the most exothermic, non-photochemical reaction available to biology. The energy liberated in this reaction can be used to activate inert bonds in difficult synthetic and degradative reactions, or to drive proton pumps to create transmembrane proton gradients in aerobic respiration. An understanding of the molecular mechanism of oxygen reduction to water has thus implications to the understanding of a range of key biological processes. The aim of this study was to capture an early reaction intermediate in the oxygen reduction pathway of cytochrome cd$_1$.

Cytochrome cd$_1$ was first characterized as a soluble cytochrome oxidase (1, 2). Recent crystal structures of the dimeric enzyme from Paracoccus pantotrophus (3, 4) show that each polypeptide chain is divided into two domains. Residues 1–137 form a cytochrome c-like domain, containing a covalently bound heme, and residues 138–567 form an eight-bladed $eta$-propeller domain containing a noncovalently bound $d_1$ heme, which is the site of oxygen and nitrite reduction. The role of the heme is to shuttle electrons from periplasmic electron donors such as pseudazurin and cytochrome c$_{551}$ to the $d_1$ heme in the active site. Previous work (5) has shown that the enzyme undergoes major structural rearrangements upon reduction, and produced structures for trapped reaction intermediates in the nitrite reduction reaction of the enzyme. Based on these crystal structures, a quantum mechanical interpretation of nitrite reduction was proposed (6). It has been suggested (5, 6) that the structure of the fully oxidized enzyme (3, 4), in which the c and $d_1$ hemes show His-17/His-69 and Tyr-25/His-200 ligation, respectively, represents a resting state to which the enzyme does not necessarily return in each catalytic cycle but only when the supply of reducing equivalents is low. Recent solution studies by EPR and absorption spectroscopy (7) confirm these speculations, and show the formation of a catalytically competent form of the enzyme with “switched” c heme axial ligands (His-69/Met-106). Following complete oxidation, this structure eventually returns to the oxidized resting state. NMR studies on the isolated c domain (8) are in agreement with this suggestion.

Oxygen reduction by crystalline cytochrome cd$_1$ is a relatively fast process, and strategies worked out earlier for capturing reaction intermediates during nitrite reduction (5) could not be adopted directly. At room temperature and at atmospheric oxygen pressures, reaction intermediates did not reach high concentrations in the crystal, and the enzyme was prone to reduction by x-rays during data collection. Capturing elusive intermediates in the cytochrome oxidase reaction required other approaches in cryo-trapping and in data collection.

**EXPERIMENTAL PROCEDURES**

**Crystalization**—Cytochrome cd$_1$ from P. pantotrophus was purified (9) and crystallized (10) as described previously.

**Reaction Initiation and Cryo-trapping**—Crystals were reduced with 20 mM dithionite in a synthetic mother liquor (2.5 M ammonium sulfate, 50 mM potassium phosphate, pH 7.0). Reduction of crystals took place inside a glove box containing less than 1 ppm oxygen (Belle Technology, Bournemouth, U.K.). After reduction, excess dithionite was removed by soaking the crystals in 18% glycerol, 2.5 M ammonium sulfate, 50 mM potassium phosphate, pH 7.0. This solution freezes below $-25^\circ$C. Reduced crystals were transferred to a pressure cell (4DX Systems, Uppsala, Sweden) at $-17^\circ$C, and the oxygen pressure was raised to 15 bar. Equipment used was pre-cooled to about $-20^\circ$C temperature. After various times under pressure, the crystals were quickly (<5 s) frozen in liquid nitrogen. Frozen crystals could be stored for several months without measurable change in their spectra. Spectra were recorded at 100 K with a microspectrophotometer (4DX Systems) (11) supplied with a cold nitrogen stream (Oxford Cryosystems, Oxford, U.K.).

**Data Collection and Structure Refinement**—Data were collected on frozen crystals at 100 K on beam lines ID14 EH2 and ID14 EH3 at ESRF, Grenoble using monochromatic x-rays (wavelength 0.98 Å and 0.935 Å, respectively). Data were processed with Denzo and scaled with Scalepack (12). For statistics see Table I. A hybrid structure consisting of the N-terminal domain of the structure of oxidized P. pantotrophus cytochrome cd$_1$ (1QKS) combined with the C-terminal part of subunit A and the entire B subunit from the reduced structure (1AOF) was used.

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The atomic coordinates and structure factors (codes 1HJ3, 1HJ4 and 1HJ5) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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‡ The abbreviations used are: ESRF, European Synchrotron Radiation Facility; NO, nitric oxide; CO, carbon monoxide.
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Results and Discussion

Trapping Reaction Intermediates—Prior to exposure to oxygen, crystals of the oxidized enzyme were reduced with dithionite under anaerobic conditions in a glove box (see “Experimental Procedures”). The reduced crystals were transferred to a synthetic mother liquor solution that also contained 18% glycerol as a cryoprotectant. This solution could be cooled to about −25 °C before freezing. To slow down oxygen reduction by the crystalline enzyme and to assure the build up of a relatively high concentration of oxygen in the crystal, experiments were performed at subzero temperatures (at −17 °C, where the mother liquor surrounding the crystal was still fluid) and at elevated oxygen pressures (15 bar). Plunging crystals exposed to oxygen into liquid nitrogen preserved potential intermediates in the cytochrome oxidase reaction. The oxidation state of the cd₁ heme in the freeze-quenched crystals was checked by single crystal microspectrophotometry at liquid nitrogen temperatures (Fig. 1). The two chemically identical subunits experience different crystallographic environments and have been reported to react at different rates in the crystal (5, 16). Therefore, crystal spectra were only used as first estimates of how far the reaction had proceeded in the crystal. Under the experimental conditions described here, oxygen reduction took several minutes, and an hour-long incubation of a reduced crystal with oxygen at −17 °C returned the system to the fully oxidized resting state as judged by single crystal microspectrophotometry (Fig. 1, a and c) and x-ray crystallography. Quenching the reaction after 2 min of exposure to oxygen (Fig. 1d) could trap an early intermediate.

Spectra of flash frozen crystals remained unchanged at 80 K over several months. However, x-ray data collection on reactive oxygen intermediates is not a trivial task because electrons liberated by x-ray photons during data collection can reduce heme centra even at liquid nitrogen temperatures (Fig. 1, see also Ref. 17).²

Data Collection Strategy—A consideration of the elastic, inelastic, and photoelectric cross-sections of atoms present in protein crystals suggests that an average of 20 electrons are liberated in the sample through photoelectric and Auger emissions for each elastically scattered photon when a beam of 12 keV x-rays (~1 Å wavelength) is scattered from a protein crystal (18, 19). Photoelectrons ejected this way can travel long distances, spanning several unit cells in a protein crystal, before reaching thermal equilibrium and further electrons may be ejected during this process. Thermализed electrons can be captured by electron-depleted atoms/ions within the crystal. Effective electron traps in cytochrome cd₁ crystals are the oxidized metal centra and the active oxygen intermediates that may be present in the active site at the start of data collection. In addition, redox enzymes have evolved to funnel “loose” electrons effectively into an oxidized active site.

Fig. 1, e and f, shows spectral changes associated with the x-ray-induced reduction of the cd₁ heme irons in crystalline cytochrome cd₁. The process can readily be monitored by single-crystal microspectrophotometry. The data show that at low x-ray doses, the redox state of the heme centra was only slightly affected (Fig. 1e), but at high x-ray doses (at doses required for the collection of a full diffraction data set), a full reduction of the metal centra took place (Fig. 1f). To minimize x-ray-induced reduction, data were collected on several crystals (11 total), each with a different starting angle, and a composite data set was later assembled from the first 10° of data from each crystal. For data collection and refinement statistics see Table I.

The Bound Dioxygen Species on the cd₁ Heme—Using techniques described above, an early intermediate, corresponding to a bound dioxygen species in the cytochrome oxidase reaction of the enzyme, could be captured (2-min incubation in 15 bar oxygen at −17 °C) and observed in the crystal at 1.6 Å resolution (Fig. 2A). The omit map calculated from the composite data set using the refined structure (Protein Data Bank accession number 1HJ3) with the dioxygen molecule removed for phase calculation shows well resolved density for the bound dioxygen species. This density extends from the cd₁ heme toward the catalytically important His-388 and His-345 residues (3, 5, 6). An unrestrained refinement of the enzyme-dioxygen complex suggests that the Fe-O distance is 1.8 Å with an Fe-O-O bond angle at 134°. The distal oxygen atom of the bound dioxygen species forms hydrogen bonds with His-388 and His-345. The structure also shows a strong puckering of the cd₁ heme in the dioxygen complex.

The Oxidation State of the cd₁ Heme in the Complex—In the fully oxidized resting enzyme (3, 4), the porphyrin ring of the cd₁ heme is nearly planar. In the reduced enzyme (5), the cd₁ heme is puckered. This feature can be used as an internal indicator for the oxidation state of the cd₁ heme. Upon reduction of cytochrome cd₁ in the crystal, Tyr-25, which ligates the cd₁ heme in

² G. Carlsson, A. Berghuord, and J. Hajdu, unpublished data.
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The data collection strategy described above allowed us to visualize an early intermediate in the reduction of dioxygen by cytochrome cd₁. For a comparison, Fig. 2B shows what happens if 100° of x-ray data are collected from one of the crystals used to assemble the composite data set from which the map in Fig. 2A was calculated (see also Fig. 1, f–f). The 1.6 Å structure (Protein Data Bank accession number 1HJ4) shows that the dioxygen complex present in the crystal early in data collection (Fig. 2A) has broken down during 100° of x-ray exposure. The electron density map of Fig. 2B reflects a mixture of structures, including the original dioxygen species, a putative mono-oxygen species, and probably also a water/hydroxyl ion above the heme. The results indicate that small-scale movements of ligands and side chains may occur during x-ray data collection even at liquid nitrogen temperatures (see Refs. 21 and 22); however, the frozen state prevents larger structural rearrangements of the protein in the crystal.

**Full Reduction of Dioxygen to Water in the Crystal**—The dioxygen species captured in Fig. 2A turns over in the crystal both at 100 K (during data collection) and at 256 K (during prolonged incubation in an oxygen atmosphere). Fig. 2C shows the 1.46 Å structure (Protein Data Bank accession number 1HJ5) of the active site of a crystal, which was exposed for 60 min to 15 bar pressure of dioxygen at −17 °C (see also Fig. 1c), and then flash frozen in liquid nitrogen for data collection. The originally oxidized iron centra (Fig. 1c) in the crystal became reduced during data collection (not shown). However, the glass state of the surrounding solution prevents the large structural rearrangements (characteristic for the reduced enzyme) at 100 K in the crystal; the structure shows His-17/His-69 ligation for the c heme (characteristic for the resting oxidized c heme) and Tyr-25/His-200 for the d₁ heme (characteristic for the resting oxidized d₁ heme). These findings suggest that at the end of the catalytic cycle, the enzyme returned to a fully oxidized resting state (Fig. 1c) before it was flash frozen in liquid nitrogen. These

### Table I

**X-ray data collection and refinement statistics**

| Data set 1: dioxygen complex | Data set 2: mixed state due to x-ray reduction | Data set 3: reoxidized enzyme |
|-----------------------------|------------------------------------------------|-----------------------------|
| Data collection             | ESRF ID14 EH2                                  | ESRF ID14 EH3                | ESRF ID14 EH3                |
| Number of crystals          | 11                                             | 1                           | 1                           |
| Amount of data collected per crystal | 10 °                                           | 100 °                       | 100 °                       |
| Cell                        | a = 107.5 Å, b = 61.0 Å, c = 100.5 Å, β = 112.2 ° | a = 106.8 Å, b = 60.9, c = 100.6 Å, β = 112.1 ° | a = 106.8 Å, b = 61.0, c = 100.3 Å, β = 112.2 ° |
| Resolution range            | 30—1.6 Å (1.66—1.60)                           | 30—1.6 Å (1.66—1.60)        | 30—1.46 Å (1.52—1.46)       |
| No. of reflections          | 135,637                                        | 149,315                     | 189,123                     |
| Average intensity (I/σ)     | 14.1 (2.9)                                     | 14.2 (2.8)                  | 10.2 (2.5)                  |
| Rmerge                      | 4.6% (17.5%)                                   | 4.1% (20.2%)                | 5.0% (19.9%)                |
| Completeness                | 85.3% (85.4%)                                  | 93.9% (95.3%)               | 91.3% (84.6%)               |

Statistics for the final model

| Number of residues in the asymmetric unit | Number of solvent molecules | R factor (%) | Rmerge (%) | Average B-factor, protein atoms (Å²) | Average B-factor, all atoms (Å²) | B-factor for O1 (Å²) | B-factor for O2 (Å²) | RMSD bond length (Å) | RMSD bond angles (°) | Protein Data Bank accession no. |
|------------------------------------------|-----------------------------|--------------|------------|-------------------------------------|---------------------------------|---------------------|---------------------|---------------------|---------------------|-----------------------------|
| 1086 (subunit A: 17—107, subunit B: 26—567) | 826                         | 19.6         | 21.9       | 20.5                                | 21.2                            | 21.3                 | 25.0                | 0.012               | 1.3                 | 1HJ3                        |
| 1101 (subunit A: 17—567, subunit B: 26—567) | 894                         | 20.2         | 22.6       | 21.3                                | 22.2                            | 19.5                 | 17.1                | 0.013               | 1.2                 | 1HJ4                        |
| 1118 (subunit A: 9—567, subunit B: 9—567) | 900                         | 20.2         | 22.6       | 21.3                                | 21.3                            | 19.5                 | 17.1                | 0.012               | 1.2                 | 1HJ5                        |

The resting oxidized enzyme, is released to allow substrate binding (5). Concomitantly, the c domain refolds resulting in a change in the c heme coordination from His-17/His-69 (in the oxidized resting enzyme) to His-69/Met-106 (in the fully reduced enzyme) (5, 8). The axial ligation of the heme centers can be used as additional indicators for assessing the oxidation state of the system.

Fig. 2A shows the heme plane puckering in subunit B of the enzyme. On the proximal side of the d₁ heme, the acetylate groups move toward the porphyrin ring. This movement is accompanied with a rearrangement of neighboring protein side chains and solvent molecules. The c heme of this subunit has His/Met ligation as in the fully reduced enzyme. These features suggest that both the c and the d₁ hemes of subunit B are reduced. In this case, the bound dioxygen species is most likely a neutral dioxygen molecule that has not yet been converted to other dioxygen species (e.g. superoxide or peroxide) by the enzyme. Further support for this conclusion is the Fe-O-O bond angle of 134° in the complex. We note that electron transfer is usually faster than heme-religation, and therefore, a certain possibility exists that the actual oxidation state of these metal centers may differ from what they seem to be from the structure (7, 8, 20).

Single crystal microspectrophotometry indicates a mixture of oxidation states for the heme centers in the crystal. The two chemically identical subunits (A and B) experience different crystallographic environments in the monolclinal crystal (the asymmetric unit contains the entire dimeric enzyme), and the two subunits have different reactivities in the crystal (5). This seems to be the case in the present study also. The structure of subunit A has His/His axial ligands for the c heme, and sports a planar d₁ heme on which density for Tyr-25 is connected to the d₁ heme iron (not shown). This suggests that the A subunit is fully oxidized in the complex and has probably completed its catalytic cycle before the sample was flash frozen.

The data collection strategy described above allowed us to visualize an early intermediate in the reduction of dioxygen by cytochrome cd₁. For a comparison, Fig. 2B shows what happens if 100° of x-ray data are collected from one of the crystals used to assemble the composite data set from which the map in Fig. 2A was calculated (see also Fig. 1, f–f). The 1.6 Å structure (Protein Data Bank accession number 1HJ4) shows that the dioxygen complex present in the crystal early in data collection (Fig. 2A) has broken down during 100° of x-ray exposure. The electron density map of Fig. 2B reflects a mixture of structures, including the original dioxygen species, a putative mono-oxygen species, and probably also a water/hydroxyl ion above the heme. The results indicate that small-scale movements of ligands and side chains may occur during x-ray data collection even at liquid nitrogen temperatures (see Refs. 21 and 22); however, the frozen state prevents larger structural rearrangements of the protein in the crystal.
results demonstrate that the crystalline enzyme can complete a catalytic cycle in the cytochrome oxidase reaction at \(-17 ^\circ C\) and is capable of returning to a fully oxidized resting state at the end of this reaction before flash freezing in liquid nitrogen.

Functional Implications—Oxygen is a highly toxic chemical, and when it appeared in the atmosphere a couple of billion years ago, organisms that could convert dioxygen to less harmful compounds had an evolutionary advantage. A link between oxygen-reducing enzymes and denitrifying enzymes has been proposed (23, 24), suggesting that higher cytochrome oxidases have evolved by “tinkering with denitrifying enzymes” (25).

The present study establishes that the productive binding of dioxygen (Fig. 2A) in the active site is very similar to the productive binding of nitrite (5), and suggests that the catalytic mechanisms of oxygen reduction and nitrite reduction are closely related.

Another aspect concerns the role for heme ligand switching in the function of cytochrome cd\(_1\). A depletion of reducing equivalents may arise in the periplasm at high oxygen concentrations. Under those conditions dioxygen or peroxides may react with an unprotected d\(_1\) heme iron and could create harmful radical species. Shutting off access to the d\(_1\) heme by a return to a resting form in which the d\(_1\) heme is shielded prevents the heme iron from reacting with dioxygen in an uncontrolled manner. If, however, there is a good supply of external reducing equivalents, the active site stays open, and dioxygen is reduced to water through the cytochrome c oxidase activity of the enzyme (8).

Several structures of complexes of \textit{P. pantotrophus} cytochrome cd\(_1\) with diatomic ligands have been determined. Fig. 3 shows a comparison of the dioxygen complex described here and the recently published structures of the reduced enzyme complexed with CO (16) and CN\(^{-}\) (26). A structure of an NO complex obtained by soaking a reduced crystal in nitrite (5) is also shown. A recent paper (16) shows that the binding of the neutral carbon monoxide molecule to the active site of cytochrome cd\(_1\) is accompanied by the rapid release of a proton from the enzyme. This process is reversible, and the release of the bound CO molecule from the d\(_1\) heme is followed by the uptake of a proton from bulk solvent. This is a curious phenomenon, which may also accompany the binding of other neutral diatomic ligands such as dioxygen to the active site. If the routes...
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between His-388 and His-345 is taken up by the side chain of Tyr-25. The existence of dual conformations for NO is thought to be important for effective product release (5, 6).

Structural results presented here provide insight into oxygen chemistry in cytochrome cd₁ and on heme centra in general. The methods described in this paper are also applicable to other systems including higher oxidases.

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Fig. 3. Comparison of known complexes of cytochrome cd₁ from P. pantotrophus with diatomic ligands. A, the dioxygen complex at 1.6 Å resolution as described in this study (Protein Data Bank accession number 1HJ3, subunit B). B, reduced enzyme complexed with CO at 1.57 Å resolution (Protein Data Bank accession number 1DY7, subunit B) (16). C, reduced enzyme complexed with cyanide at 1.59 Å resolution (Protein Data Bank accession number 1E2R, subunit B) (26). D, NO complex at 1.8 Å resolution on an oxidized heme obtained by soaking a reduced crystal in nitrite solution (Protein Data Bank accession number 1A0Q, subunit B) (5).
Structure of the Bound Dioxygen Species in the Cytochrome Oxidase Reaction of Cytochrome \textit{cd} \textit{I} Nitrite Reductase

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