Biochimica et Biophysica Acta 1847 (2015) 79–85

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Review

Time-resolved infrared spectroscopic studies of ligand dynamics in the active site from cytochrome *c* oxidase ☆

Marten H. Vos, Ursula Liebl *

Laboratory of Optics and Biosciences, CNRS, Ecole polytechnique, 91128 Palaiseau, France
INSERM U696, 91128 Palaiseau, France

ARTICLE INFO

Article history:
Received 27 May 2014
Accepted 29 July 2014
Available online 9 August 2014

Keywords:
Ligand dynamics
Cytochrome *c* oxidases
Heme proteins
Binuclear center
Time-resolved spectroscopy

ABSTRACT

The catalytic site of heme–copper oxidases encompasses two close-lying ligand binding sites: the heme, where oxygen is bound and reduced and the Cu_B atom, which acts as ligand entry and release port. Diatomic gaseous ligands with a dipole moment, such as the signaling molecules carbon monoxide (CO) and nitric oxide (NO), carry clear infrared spectroscopic signatures in the different states that allow characterization of the dynamics of ligand transfer within, into and out of the active site using time-resolved infrared spectroscopy. We review the nature and diversity of these processes that have in particular been characterized with CO as ligand and which take place on time scales ranging from femtoseconds to milliseconds. These studies have advanced our understanding of the functional ligand pathways and reactivity in enzymes and more globally represent intriguing model systems for mechanisms of ligand motion in a confined protein environment. This article is part of a Special Issue entitled: Vibrational spectroscopies and bioenergetic systems.

1. Introduction

Cytochrome *c* oxidase (CcO) aa_3 is the terminal oxidase of cellular respiration in higher eukaryotes and is equally present in a large number of microorganisms. This enzyme complex catalyzes the four-electron reduction of molecular oxygen to water and during this process, the redox energy is stored as a transmembrane proton gradient that is subsequently used to generate adenosine triphosphate (ATP) [1,2]. On the catalytic subunit I, CcO aa_3 contains a six-coordinated low spin heme *a* that mediates electron transfer from exogenous cytochrome *c* toward the active site of the enzyme and a bimetallic center composed of the five-coordinated heme a_3 and the adjacent Cu_B ion, which is located 5 Å away (Fig. 1). Located on a different protein subunit is the redox-active copper center Cu_A that is formed by two copper atoms. Upon reduction, Cu_A holds an electron by sharing it between both copper atoms.

While cytochrome *c* oxidase aa_3 is the best known representative of the superfamily of heme–copper oxygen reductases, several different bacteria-specific representatives exist, like the caa_3 and ba_3 oxidases, probably best characterized from the bacterium *Thermus thermophilus* [3,4] or the cytochrome cbb_3 oxidases with three extra subunits besides the catalytic one; two of them containing additional redox centers, a diheme and a monoheme cytochrome [5]. Whereas this review primarily concerns ligand dynamics in CcO aa_3, these bacterial oxidases provide interesting systems for comparison. In CcO aa_3, heme a_3 is the binding site for molecular oxygen and its reaction intermediates during its reduction and also binds competitor ligands like CO and NO, which act as biological messengers. Because heme binds CO (and NO) tightly, the accessibility of these ligands to heme a_3 must be well controlled, with a possible role of Cu_B in regulating ligand traffic. Before binding to heme a_3, O_2 or other small-molecule ligands bind to Cu_B intermittently [6]. The reverse reaction, involving ligand dissociation from heme a_3 and eventual transfer out of the protein via prior binding to Cu_B, can be studied using flash photolysis, exploiting the photolability of the heme–ligand bond. As CO forms a stable complex with cytochrome *c* oxidase, it has been widely used to investigate the mechanisms, dynamics and the pathways open to oxygen and other exogenous ligands to and from the active site.

Time-resolved resonance Raman spectroscopy has been used to investigate the dynamics after CO dissociation from reduced CcO on picosecond and longer time scales, while UV–Vis absorption experiments have been performed on femtoseconds to milliseconds time scales [7,8]. These experiments probe only the heme sites directly, while the reduced Cu_B site is generally spectroscopically silent. Electronic absorption and resonance Raman studies on cytochrome *c* oxidase aa_3 are further complicated by contributions from both cytochromes *a* and a_3, which can be difficult to distinguish.

In contrast, infrared spectroscopy is uniquely suited as a probe for the reactions of CO with CcO, particularly the ligand binding reactions of Cu_B. The high oscillator strength of the CO stretch infrared absorption band around 2000 cm^{-1} combined with the high sensitivity of its frequency and bandwidth to changes in binding and protein environment,

☆ This article is part of a Special Issue entitled: Vibrational spectroscopies and bioenergetic systems.

* Corresponding author.

http://dx.doi.org/10.1016/j.bbabio.2014.07.018
0005-2728/

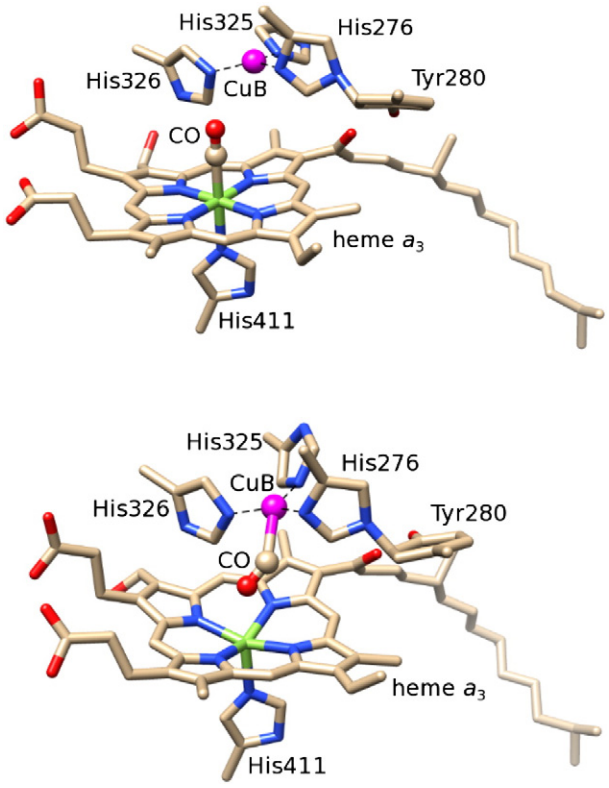

Fig. 1. Structures of the active site of CcO aa_3 from *Paracoccus denitrificans*, with CO liganded to heme a_3 (top) and Cu_B (bottom). The structures are snapshots taken from molecular dynamics simulations described in Ref. [18]. The dashed lines represent hydrogen bonds.

as well as the fact that this band stands background-free, well separated from the other vibrational transitions of the protein complex, make it an ideal probe of ligation reactions.

The structural dynamics of biological reactions in protein complexes span many orders of magnitude in time. Although the *overall* rate of O_2 turnover by cytochrome *c* oxidase aa_3 is quite moderate (~600 s^{-1}), the process encompasses ultrafast events of ligation of small molecules to the binuclear site. Different from standard descriptions of enzymatic catalysis, where the transition state between reactant and product is reached by thermal, stochastic motions, in the ultrafast time domain, the protein moiety and cofactor motions leading to altered conformations can be coherent rather than stochastic in nature. Such coherent motions may play a key role in controlling the accessibility of the transition state and explain the high efficiency of the reaction. Cytochrome *c* oxidase, with its two ligand binding sites distant of only a few Ångstrom, thus provides a unique environment in which the mechanisms of ligand transfer and bond formation can be studied directly.

2. Technical aspects

Most of the phenomena described in this review have been observed using time-resolved mid-infrared techniques. The common steady-state technique in this spectral range is Fourier Transform Infra Red (FTIR). Here, a mostly continuous, broad-band light source is used and spectral resolution is obtained using a Michelson interferometer. In this way, no losses due to dispersive or spectral selection optics occur and the entire available infrared light intensity from the, essentially black body, continuous light-source can be exploited for detection. Time resolution while maintaining spectral resolution can be obtained by coupling this technique with a pulsed visible light source (typically a nanosecond laser) that triggers a perturbation of the system. For heme proteins, and in particular here heme–copper oxidases, this optical perturbation can result in the dissociation (photolysis) of a ligand bound to the heme in the active site, in particular for CO or NO as ligands. Recently, electrochemical pulses have also been explored in combination with surface-enhanced IR spectroscopy to study the protein response to electron transfer events [9].

For processes slower than the scan time, full interferograms at different times after the perturbation can be measured (rapid scan); for higher time-resolution the inverse approach is taken and kinetics at different interferogram positions are taken, allowing full recovery between scan steps (step scan) [10,11]. The latter method has a time resolution in principle in the nanosecond range, limited by the detector response and the laser pulse length. It is available on several commercial instruments and has been used for a number of studies on ligand dynamics near the active site of CcO.

If spectral resolution is not required, intense continuous monochromatic infrared sources can be used for probing. For instance, Dyer and coworkers used a tunable IR diode laser in early experiments on the microsecond kinetics of the Cu_B-bound CO complex in CcO [12].

For subnanosecond resolution, ultrashort visible excitation and infrared detection pulses are used and time resolution is achieved by mechanical scanning of the delay between these pulses (pump-probe principle). Here, with femtosecond pulses, intense infrared pulses with spectral widths up to hundreds of cm^{-1} and temporal widths in the range of 10–200 fs can be generated using non-linear optical techniques like difference frequency generation [13] or optical rectification [14]. Spectral resolution of the probe pulse can be obtained by dispersion in an infrared polychromator and detection with (typically 64-pixel) infrared detector arrays [15]. Alternatively, interferometry can also be used for spectral resolution [16]; note that in this case the interferometric scan range (10 ps full range for a 3 cm^{-1} spectral resolution) can contribute to the effective temporal resolution. A promising new method for spectrally resolving short infrared pulses is its upconversion to the visible domain by mixing with a chirped near-infrared pulse, and subsequently detecting it after dispersion using a charge coupled device (CCD) [17]. The higher pixel density and dynamic range of these less costly detection devices allow high spectral resolution and sensitivity. This method has been used to temporally resolve the femtosecond transfer dynamics of CO between the two binding locations in the CcO active site [18].

We note that the high yields of infrared light generation by downconverting ultrashort visible light can also been exploited for experiments with low time resolution, avoiding the need for interferometric spectral characterization. This approach has been applied in recent work on the protein response to CO dissociation from Cu_B in bovine heart CcO, where the delay between a nanosecond visible pulse and a broad-band femtosecond infrared probe pulse was electronically controlled, and the probe beam, highly attenuated by a strongly absorbing background, was spectrally resolved via dispersion and detector arrays [19].

Exploiting visible transient absorption spectroscopy, for example in colored heme proteins, low frequency molecular vibrations can in principle also be observed in real time as coherent modulations of the spectra [20,21]. This method has been used to demonstrate ballistic population transfer during CO migration within the CcO binuclear active site [22].

3. Ultrafast ligand dynamics in the binuclear site of cytochrome *c* oxidases

As stated before, photodissociation studies of CO have played a major role in elucidating the ligand binding reactions of the binuclear center in cytochrome *c* oxidases. Early flow-flash experiments based on CO photodissociation from CcO aa_3, pioneered by Gibson and Greenwood [23], established the first kinetic evidence for short-lived binding intermediates [24].