Earlier data has shown that the E122, E125, E198 and E267 are essential for activity, but not assembly, whereas the E202 is not essential for activity. We present single turn-over data (on fully reduced NOR and oxygen) on alanine mutants of all five conserved glutamates. Our data show that, except the E202, they are all crucial for the oxidative phase of the reaction which is limited by proton uptake to the active site. Together with a model of the NorB, we propose that the E122 and E125 sit at the entrance of the proton pathway which also contains E267 and E198, but not the E202.

doi:10.1016/j.bbabio.2008.05.286

S11.32 Single-turnover of *ba*$_3$ oxidase from *Thermus thermophilus*

Ilya Belevich[a], Sergey A. Siletsky[b], Audrius Jasaitis[c], Alexander A. Konstantinov[b], Mårten Wikström[a], Tewfik Soulimane[d], Michael I. Verkhovsky[a]
[a]*HBG, Institute of Biotechnology, University of Helsinki, Helsinki, Finland*
[b]*Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, Russia*
[c]*European Laboratory for Non-Linear Spectroscopy, University of Florence, Italy*
[d]*Materials and Surface Science Institute, University of Limerick, Ireland*
E-mail: ilya.belevich@helsinki.fi

Cytochrome *ba*$_3$ from *Thermus thermophilus* belongs to the large family of structurally related heme-copper terminal oxidases. It catalyses the process of oxygen reduction to water and couples it with creation of an electrochemical transmembrane gradient of protons, which is subsequently used for ATP synthesis. The kinetics of the oxidation of fully-reduced *ba*$_3$ oxidase by oxygen were followed by time-resolved optical spectroscopy and electrometry. Four catalytic intermediates were resolved during this reaction. The chemical nature and the spectral properties of three intermediates (A, P, O) reproduce the general features of *aa*$_3$-type oxidases. However the F intermediate in *ba*$_3$ oxidase has a spectrum identical to the P state. This indicates that the proton taken up during the P→F transition does not reside in the binuclear site but is rather transferred to the covalently cross-linked tyrosine near that site. The total charge translocation associated with the F→O transition in *ba*$_3$ oxidase is close to that observed during the F→O transition in the *aa*$_3$ oxidases. However, P_R→F is characterized by significantly lower charge translocation, which probably reflects the overall lower measured pumping efficiency during multiple turnovers.

doi:10.1016/j.bbabio.2008.05.287

S11.33 Characterization of the membrane-bound tri-heme *c* quinol peroxidase functionally connected to the respiratory chain

Eizo Takashima, Hiroyuki Yamada, Konishi Kiyoshi
Department of Microbiology, School of Life Dentistry at Tokyo, Nippon Dental University, Japan
E-mail: eizo.takashima@gmail.com

Recently, we discovered quinol peroxidase (QPO) activity, the reduction of hydrogen peroxide by ubiquinol-1 as an electron donor, from the membrane fraction of the bacteria *Aggregatibacter actinomycetemcomitans* that is closely related to *Haemophilus* and has been associated with localized aggressive periodontitis. The aim of this study is to biochemically characterize QPO. QPO was purified to >90% purity from the membrane fraction. Using the N-terminal amino acid sequence of the QPO, we identified the *qpo* gene. The amino acid sequence of QPO shared 46~54% sequence identity with gene homologues in *Escherichia coli*, *Bacteroides fragilis*, etc. QPO also has a high sequence homology to bacterial di-heme cytochrome *c* peroxidase (BCCP), but QPO did not catalyze peroxidation in the presence of horse heart cytochrome *c*. MALDI-TOF MS analysis showed that QPO is a 53.6-kDa protein that contains 3 heme *c* molecules. The Km value for ubiquinol-1 was 107 μM and the optimum pH was 7.5. The Kcat value was 582 s^{-1}, comparable to that of *Paracoccus pantotrophus* BCCP. Moreover, the membrane fraction of *A. actinomycetemcomitans* had an apparent QPO-dependent peroxidase activity in the presence of NADH and succinate. Based on these findings, we present a new mechanism for the scavenging of reactive oxygen species in which quinol in the respiratory chain is consumed.

doi:10.1016/j.bbabio.2008.05.288

S11.34 The semiquinone at the Q_H site of the cytochrome *bo*$_3$ from *Escherichia coli*

Lai Lai Yap[a], Rimma I. Samoilova[d], Myat T. Lin[b], Robert B. Gennis[a], Sergei A. Dikanov[c]
[a]*Department of Biochemistry, University of Illinois, USA*
[b]*Department of Biophysics, University of Illinois, USA*
[c]*Department of Veterinary Clinical Medicine, University of Illinois, USA*
[d]*Institute of Chemical Kinetics and Combustion RAS, Novosibirk, Russia*
E-mail: dikanov@uiuc.edu

The aim of this study was advanced pulsed EPR characterization of the semiquinone (SQ) in the high-affinity Q_H-site of the cytochrome *bo*$_3$ ubiquinol oxidase. Our studies have shown that a SQ at the Q_H site is a *neutral* species in the wild-type protein, with two strong H-bonds to Asp-75 and either Arg-71 or Gln-101. Selective ^{15}N labeling of the side chain nitrogens was performed to distinguish between these two residues. Pulsed EPR studies have been extended to two mutants at the Q_H site. The D75E mutation has little influence on the catalytic activity, and the pattern of H-bonding is similar to the wild type. In contrast, the D75H mutant is virtually inactive. Pulsed EPR revealed significant structural changes in this mutant. The H-bond to Arg-71 or Gln-101 that is present in both the wild type and D75E mutant oxidases is missing in the D75H mutant. Instead, the D75H has a single, strong H-bond to a histidine, likely His-75. The D75H mutant stabilizes an *anionic* semiquinone as a result of the altered H-bond network. Either the redistribution of charge density in the semiquinone species, or the altered H-bonding network may be responsible for the loss of catalytic function.

doi:10.1016/j.bbabio.2008.05.289

S11.35 Resolution of a novel catalytic intermediate in cytochrome *bd* terminal oxidase in real time: A true peroxy species?

Vitaliy B. Borisov[a], Ilya Belevich[b], Michael I. Verkhovsky[b]
[a]*Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow, Russia*
[b]*Helsinki Bioenergetics Group, Institute of Biotechnology, University of Helsinki, Helsinki, Finland*
E-mail: bor@genebee.msu.su

Cytochrome *bd* is a terminal quinol oxidase of bacterial respiratory chains containing three hemes: b_{558}, b_{595} and *d*. Transient formation of catalytic intermediates in reaction of cytochrome *bd* terminal oxidases from *Escherichia coli* and *Azotobacter vinelandii* with oxygen was monitored by microsecond-resolved absorption spectroscopy and electrometry. Initial binding of O_2 by three-electron-reduced enzyme is followed by conversion of oxy-complex (A) to previously unresolved oxygen species,

denoted P. During A→P transition, heme b_{595} is oxidized whereas heme b_{558} remains reduced. The P formation is not coupled to membrane potential generation. Reduction of O_2 by two electrons is sufficient to produce (hydro)peroxide bound to ferric heme *d*. Hence, if O–O bond is left intact in the P state, P is a true peroxy complex of heme *d* (Fe_d^{3+}–O–O–(H)) corresponding to compound 0 in peroxidases. If O–O bond is broken, P is heme *d* oxoferryl species ($Fe_d^{4+}=O^{2-}$) with a nearby radical (most likely amino acid residue), analogous to compound I of cytochrome *c* peroxidase or P_M species of cytochrome *c* oxidase. Decay of P to oxoferryl species is accompanied by heme b_{558} oxidation and this process is electrogenic.

Work was supported by Russian Foundation for Basic Research.

doi:10.1016/j.bbabio.2008.05.290

S11.36 Crystallisation and preliminary X-ray diffraction analysis of *CAA$_3$*-cytochrome *c* oxidase from *Thermus thermophilus*

Orla Slattery[a], Martin Caffrey[a,b], Tewfik Soulimane[a,b]
[a]*Department of Chemical and Environmental Sciences*
[b]*Materials and Surface Science Institute, University of Limerick, Limerick, Ireland*
E-mail: tewfik.soulimane@ul.ie

The last step in the respiratory chain uses a proton-pumping cytochrome *c* oxidase to reduce molecular oxygen to water. The extreme thermophilic, gram negative bacterium *Thermus thermophilus* expresses two distantly related cytochorme *c* oxidases, *ba$_3$*- and *caa$_3$*-oxidase. The latter is unique among the heme-copper oxidase superfamily because it exists as a complex of the oxidase enzyme and its substrate, cytochrome *c*. The crystal structures of the *ba$_3$*-oxidase and it substrate cytochrome c_{552} have been reported to high resolution. Our current aim is to solve the structure of its *caa$_3$* counterpart. The *caa$_3$*-oxidase has been solubilised from *Thermus* membranes and purified according to an established protocol by ion exchange and gel-filtration chromatography. Purification takes approximately two weeks and yields about 10 mg purified enzyme from 100 g biomass. The purified enzyme has been characterised by UV-visible spectroscopy and SDS-PAGE. Crystals of the *caa$_3$*-oxidase have been obtained by vapour diffusion sitting drop (*in surfo*) and cubic phase (*in meso*) methods. *In meso*-grown crystals diffracted to 2.8 resolution at ID-23-2, ESRF (Grenoble) but were found to be radiation sensitive. Optimisation of crystallisation conditions and stabilisation of the crystals for data-collection are in progress. Presented here are details of the purification, characterisation, *in surfo* and *in meso* crystallisation and initial diffraction results.

doi:10.1016/j.bbabio.2008.05.291

S11.37 Ultrafast ligand binding dynamics in the active site of native bacterial nitric oxide reductase

Sofia M. Kapetanaki[a,b], Sarah J. Field[c], Ross J.L. Hughes[c], Nicholas J. Watmough[c], Ursula Liebl[a,b], Marten H. Vos[a,b]
[a]*Laboratoire d'Optique et Biosciences, CNRS, Ecole Polytechnique, F-91128 Palaiseau, France*
[b]*INSERM U696, F-91128 Palaiseau, France*
[c]*Centre for Metalloprotein Spectroscopy and Biology, School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ, UK*
E-mail: sofia.kapetanaki@polytechnique.edu

The catalytic subunit of nitric oxide reductase (NOR) from *Paracoccus denitrificans* is evolutionarily related to that of heme-copper oxidases. With the aim of exploring the interactions of external ligands with NOR, using ultrafast transient absorption spectroscopy we investigated the dynamics of the physiological substrate NO, and of CO, with the active site, which contains heme (heme b_3) and non-heme iron (Fe_B). We find that, upon photodissociation from heme b_3, 20% of the CO rebinds in 170 ps, suggesting that not all the CO transiently binds to the non-heme iron. The remaining 80% do not rebind within 4 ns and likely migrate out of the active site without transient binding to the non-heme iron. Rebinding of NO to ferrous and ferric heme takes place in ~13 ps. Our results reveal that heme-ligand recombination in this enzyme is considerably faster than in heme-copper oxidases and point at a constrained active site and (at least for CO) a low probability of transient binding to the close lying Fe_B site.

doi:10.1016/j.bbabio.2008.05.292

S11.38 The reversibility of P → F state transition in cytochrome *c* oxidase from *Paracoccus denitrificans*

Heike Angerer[a], Fraser MacMillan[b], Hartmut Michel[a]
[a]*Max Planck Institute of Biophysics, 60438 Frankfurt, Germany*
[b]*School of Chemical Sciences and Pharmacy, University of East Anglia, Norwich NR4 7TJ, U.K.*
E-mail: Heike.Angerer@mpibp-frankfurt.mpg.de

Here we demonstrate a bound peroxide ($O–O^-$) intermediate within the catalytic cycle of cytochrome *c* oxidase (C*c*O). The reaction of C*c*O from *P. denitrificans* using differing H_2O_2 concentrations provides further insight into the overall mechanism. Terminal oxidases require four electrons for cleavage of dioxygen (O=O). The **P** intermediate is an oxoferryl state implying the lack of an electron for the **R** → **P** transition. Using electron paramagnetic spectroscopy (EPR) it was shown that Y167 hosts a radical species in the H_2O_2-induced P_H state which suggests that Y167 could provide this "missing electron". While X-ray structural models of C*c*O suggest bound peroxide in the O state, optical and EPR studies indicate that other intermediates may also contain such peroxide species. Stoichiometric and excess amounts of H_2O_2 induce the $\mathbf{P}_H/\mathbf{F}_{.H}$ and F_H states, respectively and catalase-treatment of the $\mathbf{F}_H$ state leads to the apparent transition $\mathbf{F}_H$ → **P**/**F**. which is accompanied by the reappearance of an EPR signal from Y167. radical EPR signal. Here we present these *novel* $\mathbf{P}_{FH}/\mathbf{F}_{.FH}$ states and postulate that the $\mathbf{F}_H$ state hosts a superoxide (or peroxide) adduct at Cu_B (in the active site). A new model for the natural catalytic cycle is proposed incorporating the concept of a complexed peroxide bound in the **O** state.

doi:10.1016/j.bbabio.2008.05.293

S11.39 Identification of a putative quinone-binding site of the alternative oxidase

Mary S. Albury, Anthony L. Moore
Biochemistry and Biomedical Sciences, University of Sussex, Brighton, UK
E-mail: m.s.albury@sussex.ac.uk

Through accumulated data and our bioinformatics searches we have identified a region of the alternative oxidase (residues 236 to 266 of the *Sauromatum guttatum* protein) that we suggest constitutes a putative quinone-binding pocket located between α-helices II and III. Within this region we have identified six residues (Q242, N247, Y253, S256, H261 and R262) that are either totally or very highly conserved amongst all alternative oxidase sequences available to date (including plants, fungi and protists). We are using site-directed mutagenesis together with a yeast expression system