

Reactions of the reduced states of *R. sphaeroides* Cytochrome c Oxidase: an investigation using Time-resolved QCL-IR and *in-situ* ATR/FTIR spectroscopy

Federico Baserga, Pit Langner, Sven Stripp, Joachim Heberle

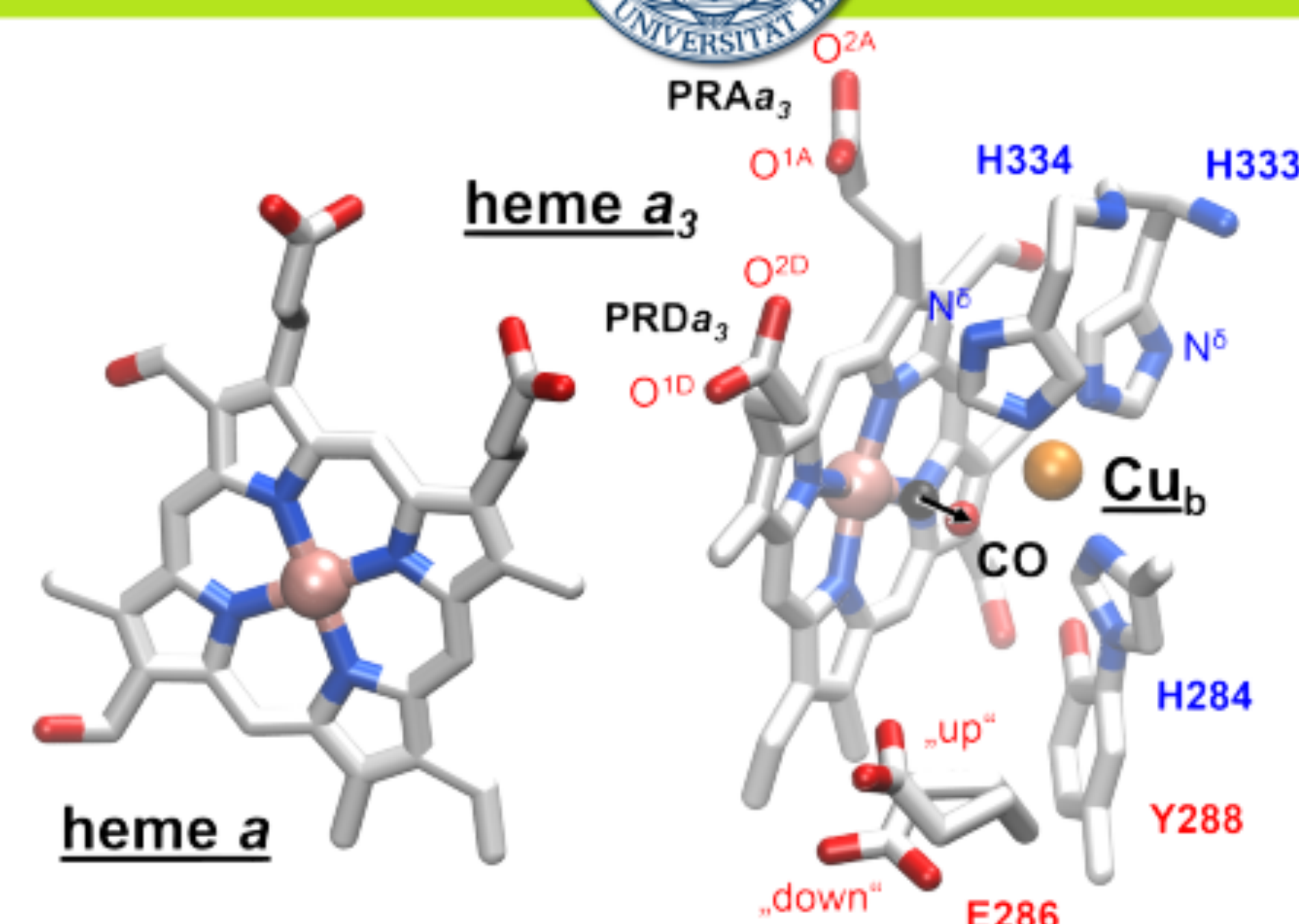


baserga@zedat.fu-berlin.de

Cytochrome c Oxidase contributes to the creation of the chemiosmotic proton gradient by reducing molecular oxygen to water and pumping protons, while undergoing a cycle initiated by electron injection from the electron-rich cytochrome c source. This cycle involves the reduction of the enzyme's metal centers and triggers a series of changes in the residues neighboring the active center. The mechanistic details of this cycle are not yet completely understood.

Using IR spectroscopy, we investigate the protonation dynamics of the enzyme. The two techniques employed are:

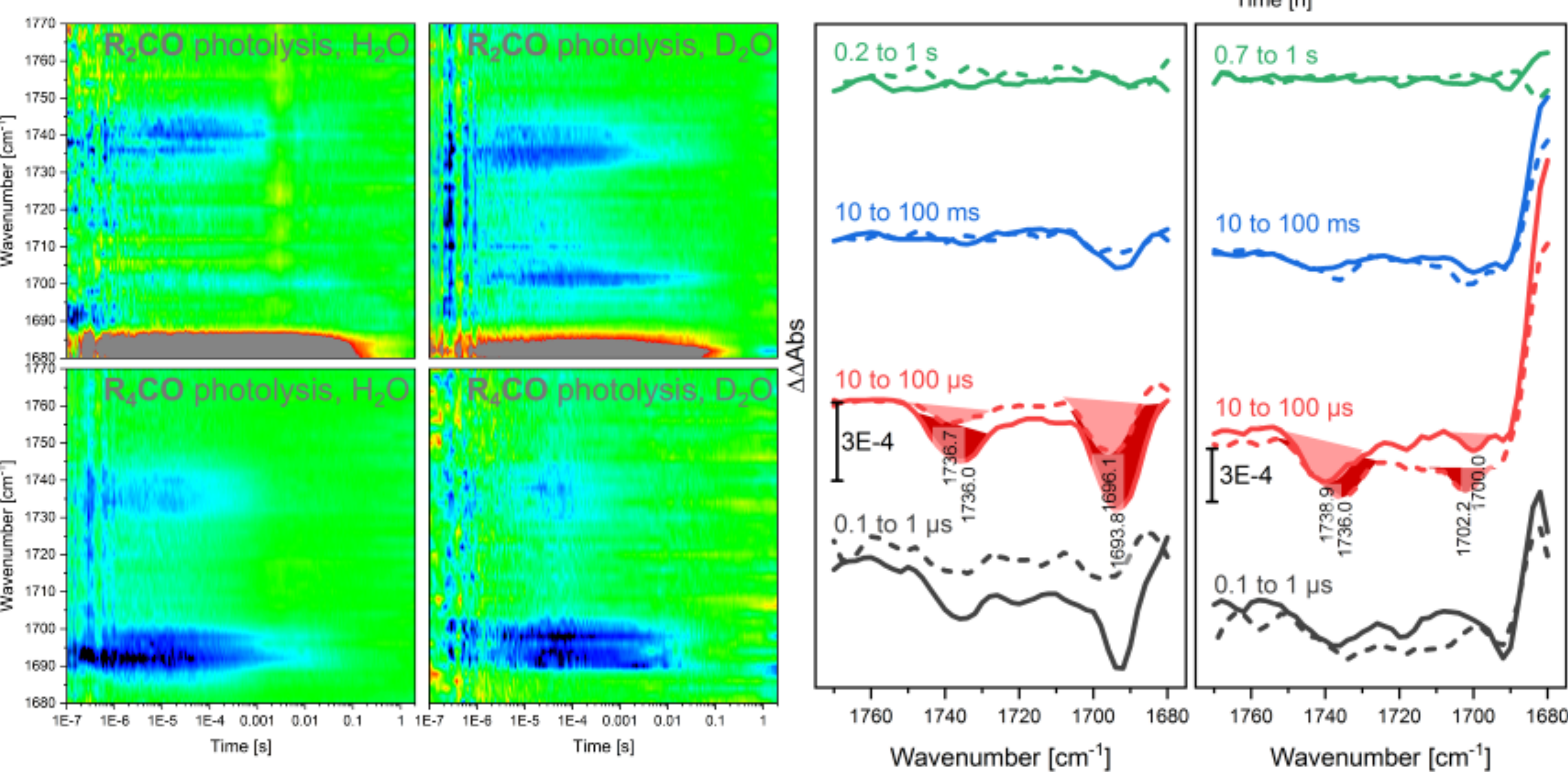
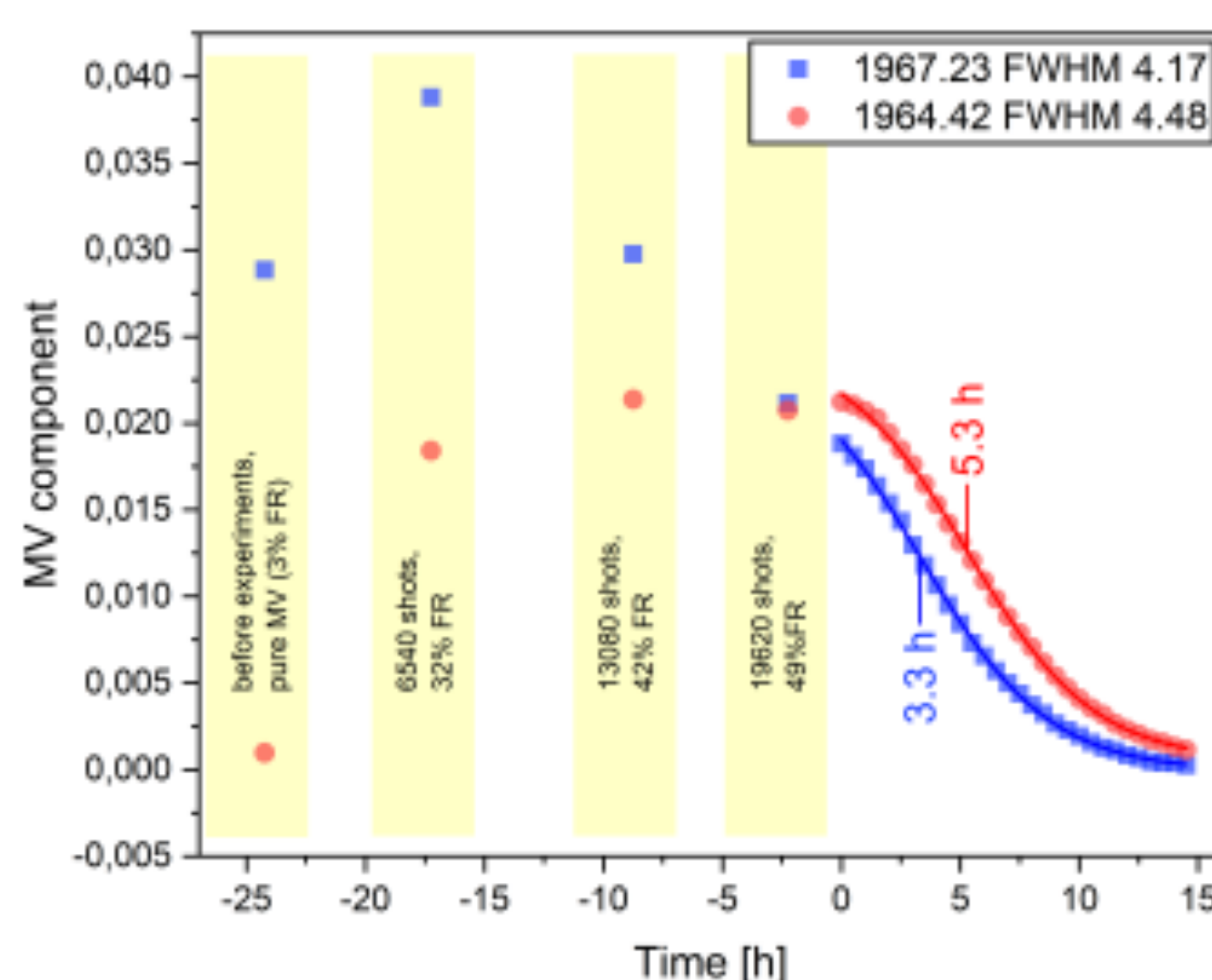
- (I) T-res IR spectroscopy, in which Carbon monoxide is used to stabilize the reduced state of the enzyme, and a Nd:YAG laser flash transiently photolyzes the CO-Fe bond;
- (II) In-situ ATR/FTIR spectroscopy, in which we prepare the enzyme in a reduced state and introduce gaseous O₂ to induce the catalytic reaction.



(I) Time-resolved IR

- Samples (CcO in ND) are prepared in a sealed vessel and undergo Vacuum/CO cycles. Reduction happens by either CO autoreduction [2], or reduction by 4 equivalents of NaDT followed by CO binding. Steady-states absolute UV/vis as well as FTIR spectra are recorded.

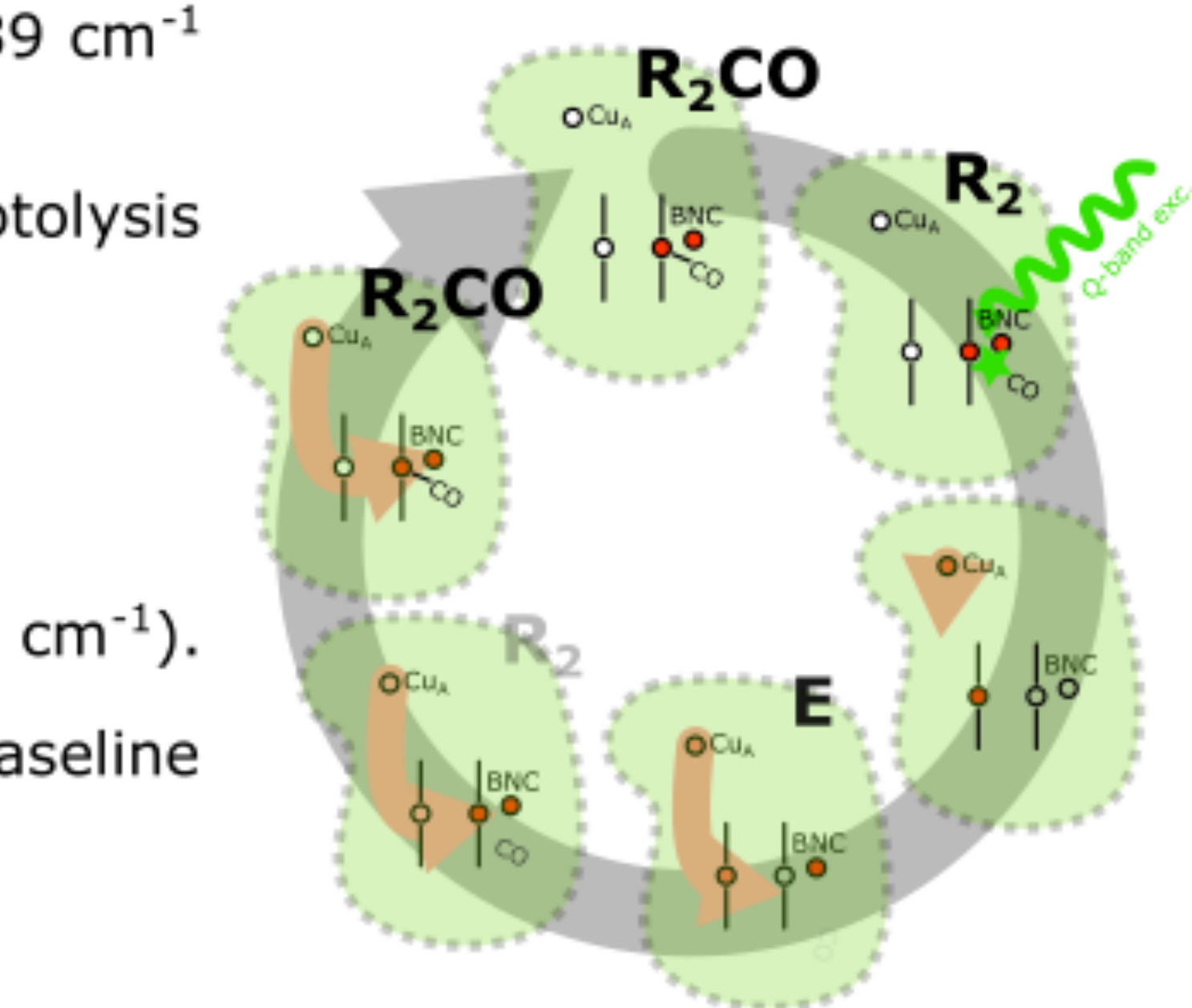
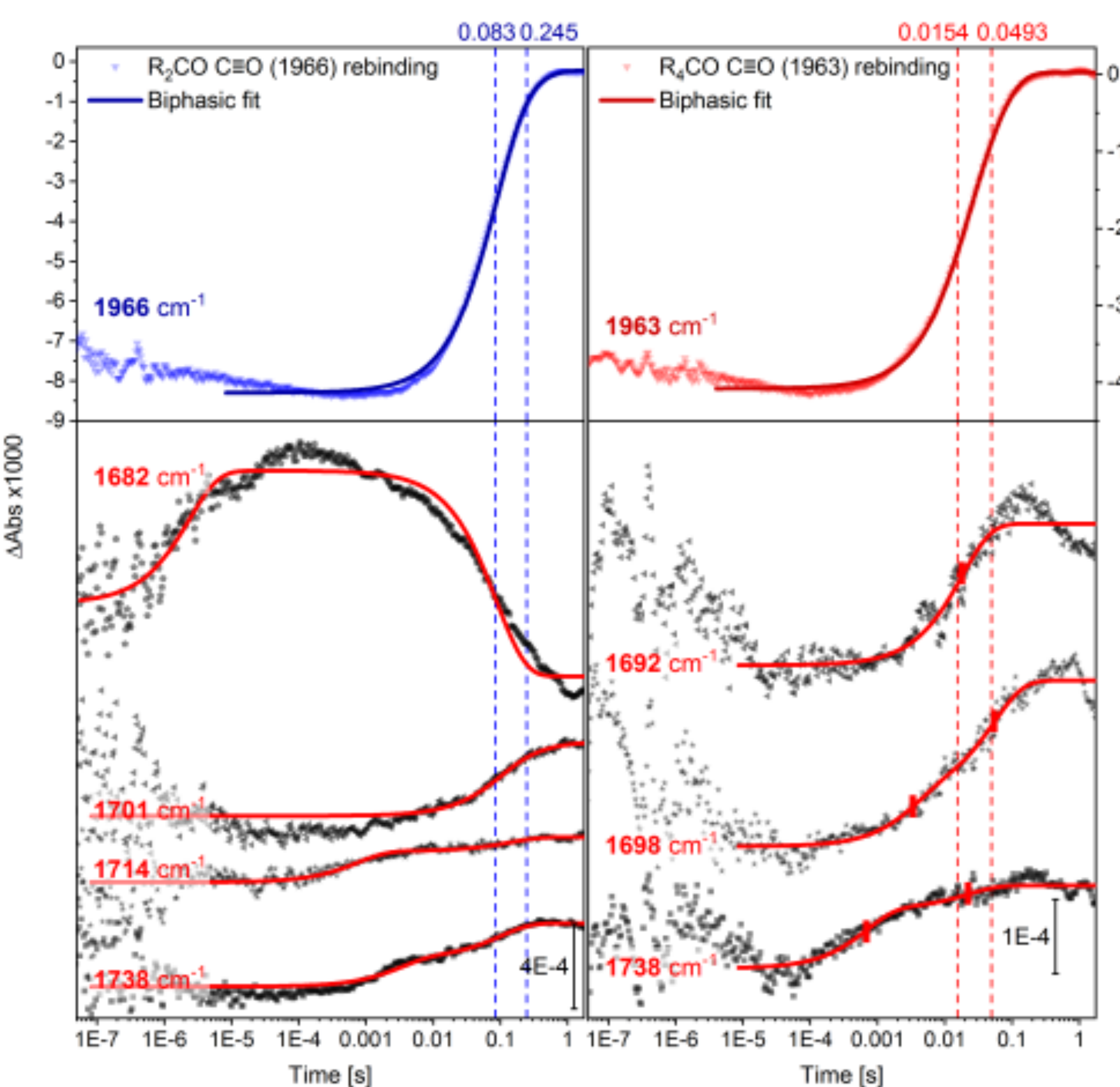
- The R₄CO state is indefinitely stable, while R₂CO slowly converts into the fully-reduced form, or decays. The internal conversion seems to be mostly due to photolytic damage.



- Bands replicate rapid scan studies such as Nyquist et al. [3]
- E286's peak is not in the usual position (1745 or 1739 cm⁻¹ in H₂O or D₂O [4]).
- CcO shows a distinct spectral response to photolysis depending if it is 4- or 2-electron-reduced.

Possible explanations:

- Failed deuteration or H₂O contamination.
- Wrong peak fit due to poor scanning resolution (2 cm⁻¹).
- Artifacts introduced by baseline correction, or baseline correction necessary in D₂O as well.

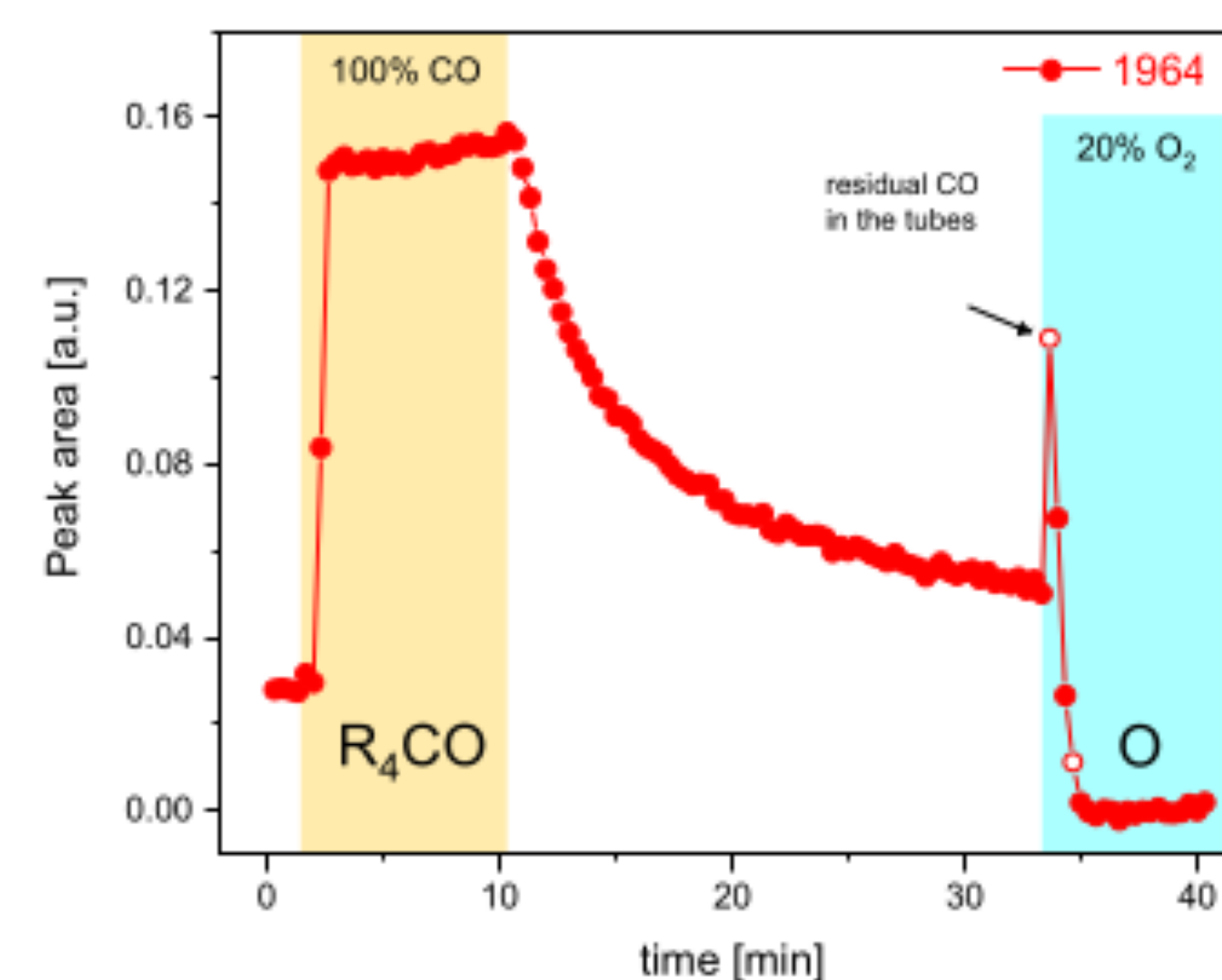


Conclusions:

- E286 shows only negative bands. This is expected for the E (probably) and P_m (definitely) states [3], while other states show E286 only changes its H-bonding environment.
- Our time resolution allows to observe the formation of the E state. What is the state before E?
- There is definitely a reaction preceding CO rebinding. This implies at least causality: the E state changes the BNC redox state, leading to formation of the covalent Fe-C≡O bond.
- The photolysis reaction of R₄CO needs a new mechanistic interpretation of the electron back-reaction.

(II) In-situ ATR/FTIR

- CcO in ND is dried on Si ATR and subsequently rehydrated by aerosolized buffer. The carrier gas is chosen between N₂, CO, dry air (20% O₂). Differently to perfusion, this is a slower reaction but results in higher S/N.



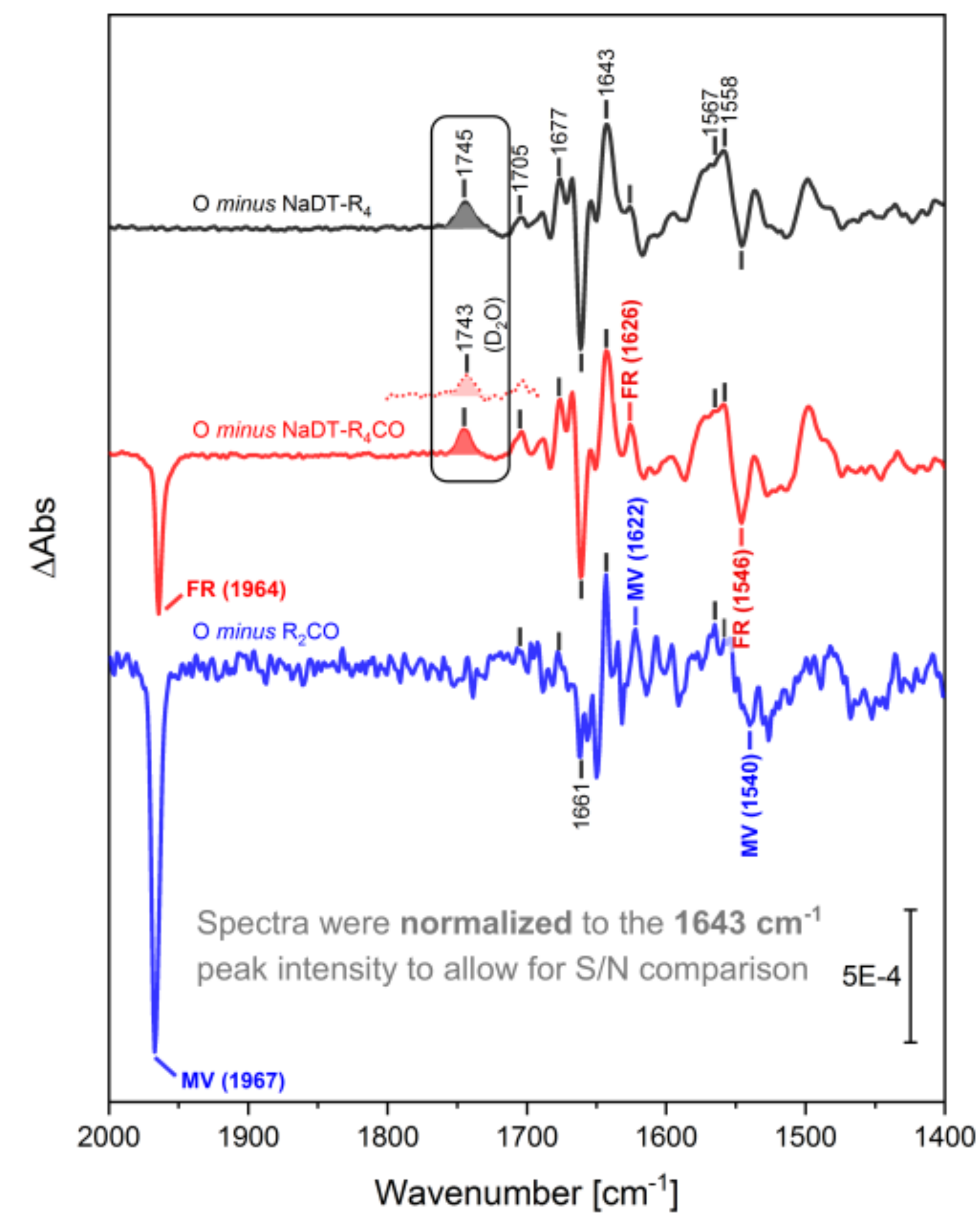
- The sample is reduced either by Na-Asc, NaDT, CO [2], or combinations thereof.

- The reaction is triggered through applying oxidizing conditions by the natural ligand O₂. Since more than 4 equivalents of reductants are present (2 mM NaDT for 100 μM CcO), samples can undergo multiple catalytic turnovers.

- The CO bound states carry their ν(C≡O) marker. Spectra show that it is possible to accumulate R₄, R₄CO, R₂CO. S/N in R₂CO is too low to comment on the state of carboxylic residues.

- The presence of CO does not vary the spectra between 1750-1400 cm⁻¹, since almost all bands are due to cofactors.

- Strangely, E286 only shows protonation between R₄ and O. Possibly, this is due to the lack of IR data showing spectra under catalytic turnover and might indicate that O₂ itself controls vectorial proton transfer, as well as the hemes' redox states.



References:

- [1] Belevich & Verkhovski, 2008, Antiox. Red. Sig., 10(1), pp.12-9. [2] Greenwood et al., 1974, Biochemical Journal, 137(2), pp.205-215. [3] Nyquist et al., 2003, PNAS, 100(15), pp.8715-8720. [4] Nyquist et al., 2001, FEBS letters, 505(1), pp.6367