T-CYCLE EPR Development at 275 GHz for the study of reaction kinetics & intermediates

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Venturing on the reoxidation of T1D SLAC with Temperature-Cycle EPR

5.1 Introduction

Temperature-Cycle EPR is a novel technique recently proven to be robust and flexible in the investigation of chemical dynamics down to the millisecond time domain. In the previous Chapters the method was demonstrated by employing well-understood, simple chemical reactions involving the reduction of a nitroxide radical. Given the successes achieved, an attempt to pioneeringly apply Temperature-Cycle EPR to the study of a biologically relevant enzymatic system on the sub-second time scale is presented here.

Multi-Copper Oxidases (MCO) are oxygen-reducing enzymes deriving their name from the four copper ions they use to promote the 4-electron reduction of O_2 to H_2O, while oxidizing a broad catalog of diverse substrates [80]. In particular, laccases are a group of multidomain MCOs that catalyze the oxidation of aromatic substrates, most notably phenols. They are ubiquitously found in nature, performing physiological roles in a wide range of organisms, and find (bio)technological applications in many industrial processes as "green" biocatalysts as they can be versatilely engineered [81] [82].

Small Laccase (SLAC) is a homotrimer laccase whose structure and mechanism of catalysis are objects of ongoing studies [83] [84] [85] [86] [87] because it operates with a different mechanism as compared to that of most MCOs. It features three identical domains, each one containing – like other MCOs – one Type-1 copper ion (T1Cu) and a trinuclear cluster (TNC) composed of one Type-2 (T2Cu) and two Type-3 (T3Cu) copper ions. The SLAC crystal structure is shown in Figure 5.1, with the three domains highlighted in different colors, and the T1Cu and TNC represented as orange and yellow spheres, respectively. The T1Cu works in pair with the TNC from an adjacent domain; the former acts as the oxidation center for the substrates, while the latter binds molecular oxygen and reduces it to water with the electrons coming from the T1Cu through the bridging aminoacid residues.

The copper ions in the fully oxidized SLAC all share a d^9 electronic configuration, exhibiting an electron spin $S = {}^1/_2$ and thus being paramagnetic. The two T3Cu of the TNC are antiferromagnetically coupled and are thus EPR-silent, while the T1Cu and T2Cu show clearly distinguishable EPR spectra due to very distinct values of the A_z copper hyperfine component [81] [88]. When SLAC is fully reduced, the copper ions have a a d^{10} electronic configuration and are thus diamagnetic.

For the study of the present Chapter a particular mutant of SLAC was employed, namely the T1Cu-depleted (T1D) enzyme [85], henceforth referred to as T1D SLAC. In this mutant, the

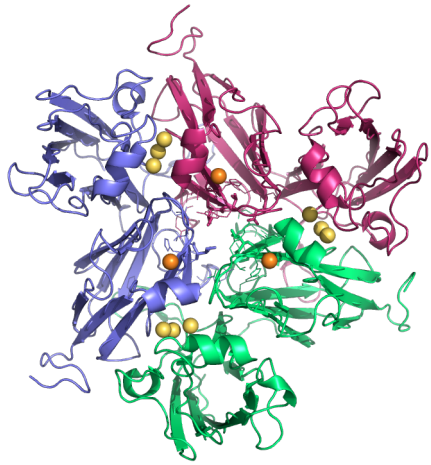

Figure 5.1: Ribbon depiction of the structure of wild-type SLAC (PDB 3CG8, from [84]). The three enzyme domains are highlighted in different colors. Type-1 copper ions are displayed as orange spheres, while Type-2 and Type-3 copper ions (forming the trinuclear copper clusters) are displayed as yellow spheres.

cysteine residue that coordinates the T1Cu in the wild-type SLAC is replaced by a serine residue, which results in an empty T1Cu site, while leaving the TNC intact. The T1D SLAC is still able to perform the reduction of O_2 to H_2O even without the T1Cu. EPR studies indicate that the role of T1Cu as electron donor in the fully reduced T1D SLAC is probably taken by a tyrosine residue, as suggested by the identification of a paramagnetic intermediate composed of a tyrosyl radical of spin $S = {}^1/_2$ in exchange interaction with the T2Cu^{2+} of the TNC [85] [86] [30]. The tyrosyl radical is estimated to be located at a distance of about 5 Å from the T2Cu, which is confirmed by crystallographic data [84]. The T2Cu–Tyr intermediate biradical couple gives rise to a triplet signal at 9.5 GHz EPR that develops within milliseconds and fades away in hours [85].

In order to avoid confusion between the different forms of oxidized and reduced T1D SLAC, the scheme of Figure 5.2 summarizes the essential states of the TNC of the enzyme when it is subject to reoxidation, and provides a simplified scheme of the reoxidation (after [89]):

- The fully oxidized, *as-synthesized* enzyme, features a paramagnetic T2Cu^{2+} (blue) axially bound to a water molecule, and two T3Cu^{2+} (light blue) antiferromagnetically coupled and thus EPR silent. A tyrosine residue (Y_{108}), which will provide an electron for the reduction of oxygen, lies in the proximity of the TNC.

- Upon reduction with sodium dithionite ($Na_2S_2O_4$), all three copper atoms of the TNC turn to the diamagnetic Cu^+ oxidation state (green), and the enzyme is in its *fully reduced* state.
- After molecular O_2 binds to the TNC, the reduced copper ions are reoxidized (which occurs in several steps), and the tyrosine residue releases an electron, becoming the tyrosyl radical (orange). This state is the enzyme's *intermediate*, featuring a $T2Cu^{2+}$–Tyrosyl biradical couple.
- As the reduction of oxygen progresses, the tyrosyl radical receives an electron and the enzyme intermediate turns to its *reoxidized* form. Notice that the as-synthesized and reoxidized forms of T1D SLAC show slight differences structurally and spectroscopically, most likely owing to a different proton arrangement at the TNC.

A first high-frequency EPR characterization of the intermediate of T1D SLAC was attempted by Nami in her PhD thesis [30], where also the development as a function of time was studied at 9.5 GHz EPR with Rapid Freeze-Quench (RFQ). In that work, however, interpretation of the spectra at 275 GHz EPR remained doubtful; furthermore, RFQ does not come free of a number of drawbacks, such as sample packing and reproducibility, and amount of material required. Considering the achievements with the Temperature-Cycle EPR technique built up in the previous Chapters of this thesis, here are reported T-Cycle EPR experiments to study the sub-second reoxidation of T1D SLAC at 275 GHz, in an attempt to prove the applicability of the method to an enzymatic system, without making use of RFQ.

5.2 Experimental

5.2.1 Materials and setup

The batch of T1D SLAC was isolated following the procedure described by Canters *et al.* [83], and dissolved in phosphate buffer (100 mM, pH 6.8) at a concentration of 4 mM (determined spectrophotometrically with the extinction coefficient $\epsilon_{280} = 48$ mM^{-1} cm^{-1}). The as-synthesized enzyme solution was stored at -80 °C until use.

Reductions of the as-synthesized T1D SLAC solutions containing 20% in volume of glycerol (Sigma-Aldrich, puriss., cat. n. 15523-1L-R) were carried out in a glove box with slight nitrogen overpressure at room temperature, with a solution of sodium dithionite ($Na_2S_2O_4$, sodium hydrosulphite 82%, Sigma-Aldrich, cat. n. 71699-250G) in slight excess concentration. In order to

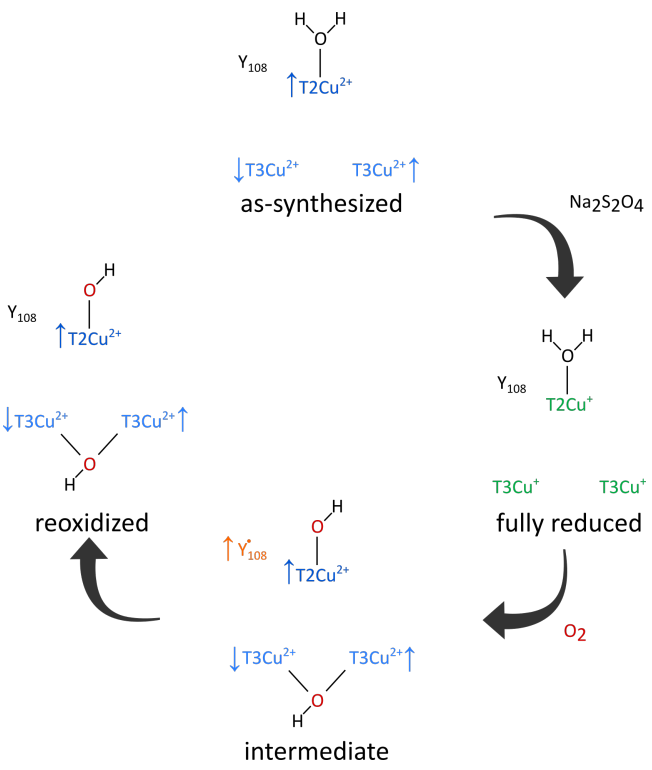

Figure 5.2: Forms of T1D SLAC in the reoxidation process, simplified from [89]: the as-synthesized (top), the fully reduced (right), the intermediate (bottom), and the reoxidized enzyme (left). Cu^{2+} ions are highlighted in blue or light blue, depending on whether they are Type-2 or Type-3, respectively; Cu^{+} ions are highlighted in green. The tyrosyl radical is highlighted in orange. The arrows next to the paramagnetic species represent an unpaired electron, and their direction indicates whether they are ferro- or antiferromagnetically coupled.

obtain a reacting mixture to be studied with T-Cycle EPR, the fully reduced sample was mixed with oxygen-saturated solutions of phosphate buffer (100 mM, pH 6.8), bubbled for one hour with oxygen, in the ratio of 1:2 in volume. The fully reduced sample was checked with 9.5 GHz EPR to ensure that no signal was present. Two samples were prepared by differently mixing the fully reduced enzyme and the oxygen-saturated buffer:

- a sample hand-mixed at room temperature with final T1D SLAC concentration of 800 μM and glycerol 7% in volume. This percentage of glycerol is obtained by mixing one part of enzyme solution containing 20% in volume glycerol, with two parts of oxygen-saturated

buffer without glycerol. Upon manual mixing at room temperature in nitrogen atmosphere (which took about two minutes), the solution was immediately frozen and stored in liquid nitrogen until needed. This sample is henceforth referred to as "RT-mixed sample", and is described in Subsection 5.3.1;

- a sample mixed with the "sub-zero mixing" technique described in Chapter 4, with final T1D SLAC concentration of 800 μM and glycerol 50% in volume. This percentage of glycerol is obtained by mixing one part of enzyme solution, having 20% in volume glycerol, with two parts of oxygen-saturated buffer with 65% in volume glycerol. A glycerol content of 20% is such that at the temperatures described for the sub-zero mixing in Chapter 4 (namely, between -50 and -60 °C), the enzyme solution is solid. For this reason, the sub-zero mixing took place at a temperature of about -30 °C, obtained thanks to a cold atmosphere produced by blowing a flow of cold nitrogen gas on a layer of liquid nitrogen. The mixture spent up to 30 minutes at that temperature due to the procedure to pack it in the capillaries for the measurement. This sample is henceforth referred to as "cryo-mixed sample", and is described in Subsection 5.3.2.

The samples for 275 GHz EPR measurements were handled, prepared, and loaded into the spectrometer's probe head using the same low-temperature setup and procedure as described in Chapters 2 to 4.

The EPR measurements were carried out with a commercial 9.5 GHz EPR spectrometer and a home-built 275 GHz EPR spectrometer; details of both are provided in Chapter 2 of this thesis. The EPR experimental parameters used to record the EPR spectra of the T1D SLAC samples are summarized in Table 5.1. The 9.5 GHz spectra were averaged 4 times, while the 275 GHz spectra were averaged between 9 and 16 times.

EPR freq. (GHz)	Field range (mT)	# of points	Mod. freq. (kHz)	Mod. ampl. (mT)	Time const. (s)	Conversion time (ms)	Microwave power (μW)	T (K)
9.5	250 ÷ 380	2048	100	0.5	0.3	82	100	40
275	8200 ÷ 10500	2300	1.253	0.7	1	500	1.28	10

Table 5.1: Experimental parameters of the EPR spectra at 9.5 GHz and 275 GHz. *Mod. freq.* and *Mod. ampl.* are the field modulation frequency and amplitude, respectively.

The T-Cycle hardware setup used here is the same one as described in Chapter 4 of this thesis. The duration and power of the laser pulses, and the temperatures involved here are summarized

in the scheme of Figure 5.3 and described below. In particular, since the ratio of water and glycerol is different in the two T-Cycle experiments performed here, in order to take the sample always to the temperature of +20 °C, the laser power and the preparation temperature (T_{prep}) employed had to be adjusted from case to case. It should be noticed that in both experiments the measurement temperature (T_{meas}) is 10 K, while the T_{prep}'s are more than 230 K higher: as opposed to the experiments described in Chapter 3 and 4 of this thesis, where T_{meas} and T_{prep} coincide, in this case it thus takes a certain amount of time (about one hour and a half) to reach the T_{prep} from the T_{meas}. From the moment the sample reaches the designated temperature and the moment the laser is turned on, an interval of less of half a minute elapses. The same applies when cooling down again to 10 K after the laser irradiation, which in total takes about an hour.

In the T-Cycle experiment described in Subsection 5.3.1 (shown in Figure 5.3 A), involving a 10 min continuous laser irradiation of the RT-mixed T1D SLAC sample containing as little as 7% in volume glycerol, a T_{prep} of -20 °C was selected, and a nominal laser power of 3.75 W. These were chosen after the laser calibration of Chapter 4: there it is shown how, with a power of 3.75 W and a composition of water and glycerol 1:1 in volume, a T-jump of about 50 °C is obtained. However, in [24] it is reported that in the absence of glycerol (i.e., a condition similar to the RT-mixed T1D SLAC sample), the laser-induced T-jumps are about 25% smaller than in the presence of 50% in volume glycerol. A T-jump of $\sim$ 40 °C is thus expected in this case, rather than of $\sim$ 50 °C derived from the previous Chapter. The final reaction temperature (T_r) of the sample is thus about +20 °C.

The T-Cycle experiment described in Subsection 5.3.2 involves two kinds of T-jump, applied on the cryo-mixed T1D SLAC sample: one induced by a laser pulse of the duration of 100 milliseconds (Figure 5.3 B), and one induced by continuous laser irradiation for the duration of 10 minutes, just like the one of the previous experiment (Figure 5.3 A). Since the sample of this experiment contains 50% in volume glycerol and the setup is identical to that described in Chapter 4, the same power calibration applies here. Therefore, for the 100 ms pulse a T_{prep} of -30 °C was selected, and a nominal power of 4.4 W; while for the 10 min continuous irradiation, a T_{prep} of -20 °C was selected, and a nominal power of 3.75 W. In both cases, the sample reaches a final T_r of +20 °C, although for the 100 ms pulse, this is achieved for a time much shorter than the duration of the pulse itself.

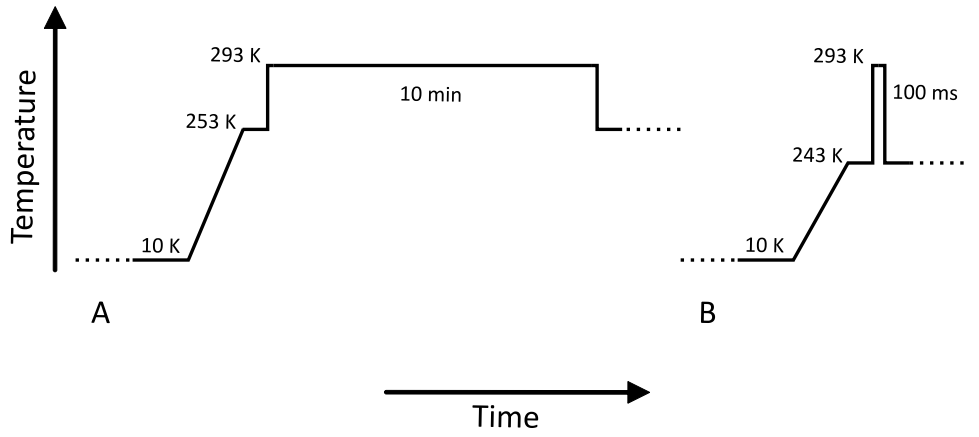

Figure 5.3: Scheme of the T-Cycle experiments applied. Both (A) and (B) feature the same measurement temperature (10 K) and reaction temperature (293 K). In (A) the preparation temperature is 253 K, the nominal laser power is 3.75 W, and the laser duration is 10 minutes. In (B) the preparation temperature is 243 K, the nominal laser power is 4.4 W, and the laser duration is 100 milliseconds. Notice that in both cases the time to reach the preparation temperature from 10 K is about 1.5 hour, and the time to reach again the temperature of 10 K from the preparation temperature is about 1 hour.

5.3 Results

In this section the EPR spectra at 9.5 and 275 GHz associated to the intermediate of T1D SLAC arising from the RT-mixed sample are reported, along with the spectrum at 275 GHz obtained after taking the RT-mixed sample to room temperature with a long laser-induced T-jump. For comparison, the spectrum of the fully oxidized T1D SLAC sample is also presented at the EPR frequency of 9.5 GHz. Furthermore, application of T-Cycle EPR to the cryo-mixed reduced T1D SLAC sample is described, and the spectra obtained are compared with the former ones used as a reference.

5.3.1 T1D SLAC sample mixed at room temperature

Since the manual mixing of the reduced T1D SLAC with oxygen-saturated buffer took about two minutes prior to freezing, the intermediate of T1D SLAC can still be detected, as its formation occurs within milliseconds, but conversion to the reoxidized enzyme takes hours [85]. Figure 5.4 A shows the 9.5 GHz EPR spectra at 40 K of the RT-mixed T1D SLAC sample (red),

and of the as-synthesized T1D SLAC (black). By comparing with the literature [85], the red spectrum is assigned to the intermediate of T1D SLAC, while the black one is assigned to the fully oxidized as-synthesized T1D SLAC.

The same RT-mixed T1D SLAC sample studied at 9.5 GHz EPR was then measured at 275 GHz at 10 K, yielding the red spectrum of Figure 5.4 B. This spectrum, arising from the intermediate of T1D SLAC, features two peaks at $\sim$ 9.528 T and at $\sim$ 9.569 T. In order to achieve the spectrum of the reoxidized T1D SLAC (blue in Figure 5.4 B), the sample was taken to room temperature by continuously irradiating the sample at a nominal laser power of 3.75 W for the duration of 10 minutes from a preparation temperature of -20 °C. The blue spectrum of Figure 5.4 B features one single peak at $\sim$ 9.592 T, thus at a higher magnetic field value as compared to the spectrum of the intermediate. This spectrum matches with that of the reoxidized form of T1D SLAC, as confirmed by other measurements on the same system at the same frequency [88] [90], and suggests that the enzyme remains intact both after warming up from 10 K to -20 °C, and after irradiation to room temperature for a relatively long time.
Both spectra of Figure 5.4 B also feature a Mn^{2+} signal at higher magnetic field, reported in the inset. The manganese signal partly originates from the buffer, as it was later detected in the spectrum of the oxygen-saturated buffer (see Subsection 5.3.2), and partly from the Mn^{2+} ions already present in the as-synthesized enzyme. The high-field manganese signal of the spectrum of the reoxidized enzyme overlaps with a more intense, broader signal of unknown nature.

5.3.2 Application of sub-second Temperature-Cycle EPR on the cryo-mixed T1D SLAC sample

Knowing the 275 GHz spectroscopic fingerprints of the intermediate and of the reoxidized form of T1D SLAC, T-Cycle EPR was applied on the cryo-mixed sample, namely the fully-reduced enzyme mixed with an oxygen-saturated buffer solution at a temperature of about -30 °C with the "sub-zero mixing" technique. Ideally, the sub-zero mixing should yield a system where no reaction has taken place, i.e., the fully reduced T1D SLAC. However, this turned out not to be the case, as the orange spectrum of Figure 5.5 was obtained, showing similarities with the spectrum of the intermediate of Figure 5.4 B, but with the peaks at a higher magnetic field, namely around 9.546 T and 9.594 T.

A T-Cycle of three steps was applied on the above mentioned cryo-mixed sample, each step consisting of heating the sample from the measurement temperature of 10 K to the preparation temperature of -30 °C, applying a laser pulse of the duration of 100 ms and of nominal power

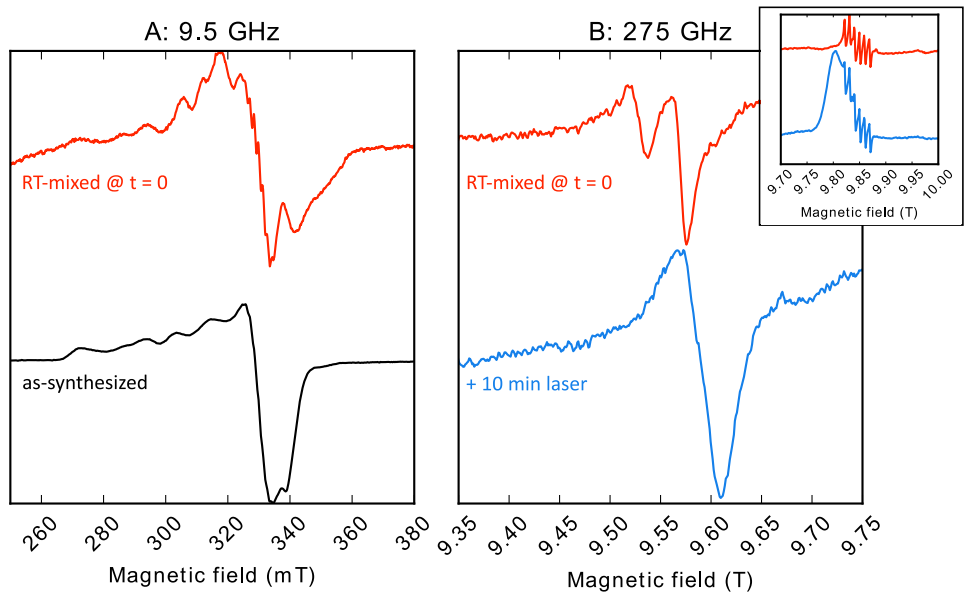

Figure 5.4: (A) 9.5 GHz cw EPR spectra at 40 K of the RT-mixed sample, i.e., T1D SLAC reduced with excess sodium dithionite and hand-mixed with oxygen-saturated buffer (red), and of the batch sample (black), i.e., the as-synthesized T1D SLAC. Notice that the intensity of the red spectrum is 6 times as small as compared to that of the black spectrum. (B) 275 GHz cw EPR spectra at 10 K of the RT-mixed sample (same sample as A), at $t = 0$ (red), and after oxidation during laser irradiation for 10 minutes at a nominal laser power of 3.75 W from a preparation temperature of -20 °C (blue). The inset shows the manganese signal of both spectra at higher magnetic field. The last one of the six peaks is used to normalize the spectra.

of 4.4 W, and cooling the sample back to the measurement temperature of 10 K to record a spectrum. Such steps are schematically represented in Figure 5.3 B. It should be noticed that this kind of experiment is time-consuming: in one working day, two to three spectra can be recorded, due to the time required to take the sample from 10 K to 243 K or 253 K (about 1.5 hours) and back (about 1 hour) – not to mention that temperature stabilization at 10 K requires between two to three hours. Because of the insufficient quality of the spectra relative to the first and third step, here only the spectrum of the second step is shown (green in Figure 5.5), corresponding to a total laser time of 200 ms. It features a poorly defined signal in the magnetic field range of 9.55 ÷ 9.65 T.

Finally, upon continuously irradiating the sample for 10 minutes at a nominal laser power of

3.75 W from a preparation temperature of -20 °C (as represented in the scheme of Figure 5.3 A), the light blue spectrum of Figure 5.5 is obtained, with a distinct feature at $\sim$ 9.596 T. The close resemblance of this spectrum to the blue one of Figure 5.4 B, obtained after a 10 min irradiation of the RT-mixed sample and corresponding to the fully oxidized T1D SLAC, points to the fact that also in this experiment the enzyme remains intact after application of several cycles of temperature changes, whether cryostat-controlled or laser-induced.

All three spectra of Figure 5.5 also contain a complex structure at higher field, with the six peaks of Mn^{2+} clearly identifiable, superimposed on a signal broad several hundreds of mT, plus a smaller signal around 9.969 T. Such structure does not seem to belong to T1D SLAC, as the spectrum of the buffer used in the enzyme solution (shown in gray in the inset of Figure 5.5) displays the same structure – with the addition of a smaller peak around 9.621 T – which might thus be either in the buffer stock solution, or present in the microwave cavity (which was however checked and found clean prior to the experiments described here).

5.4 Discussion and conclusions

T-Cycle EPR has been applied for the first time on an enzyme-catalyzed reaction, namely the reduction of O_2 to H_2O via reoxidation of T1D SLAC. The results shown in this Chapter, however preliminary, suggest that T-Cycle EPR can be successfully employed in the investigation of enzyme kinetics.

Firstly, because the strong temperature differences the sample is subject to do not lead to irreversible denaturation or other damage of the enzyme – this being a known problem associated to the protein going through temperature changes and phase transitions of the solution [91]. The enzyme can thus undergo at least a few T-Cycle steps, which involve first going from the temperature measurement of 10 K to the preparation temperature (with a ΔT of at least 230 K), and from the preparation temperature to the reaction temperature (i.e., room temperature) through laser-induced T-jumps.

Secondly, a spectral change of the cryo-mixed sample was detected on the time scale of hundreds of milliseconds. Although the interpretation of the ill-defined spectrum of the cryo-mixed sample after 200 ms laser irradiation remains tentative (because of both the baseline and the background signal), this suggests that the road towards future experiments to be performed to investigate the T1D SLAC system further is now open.

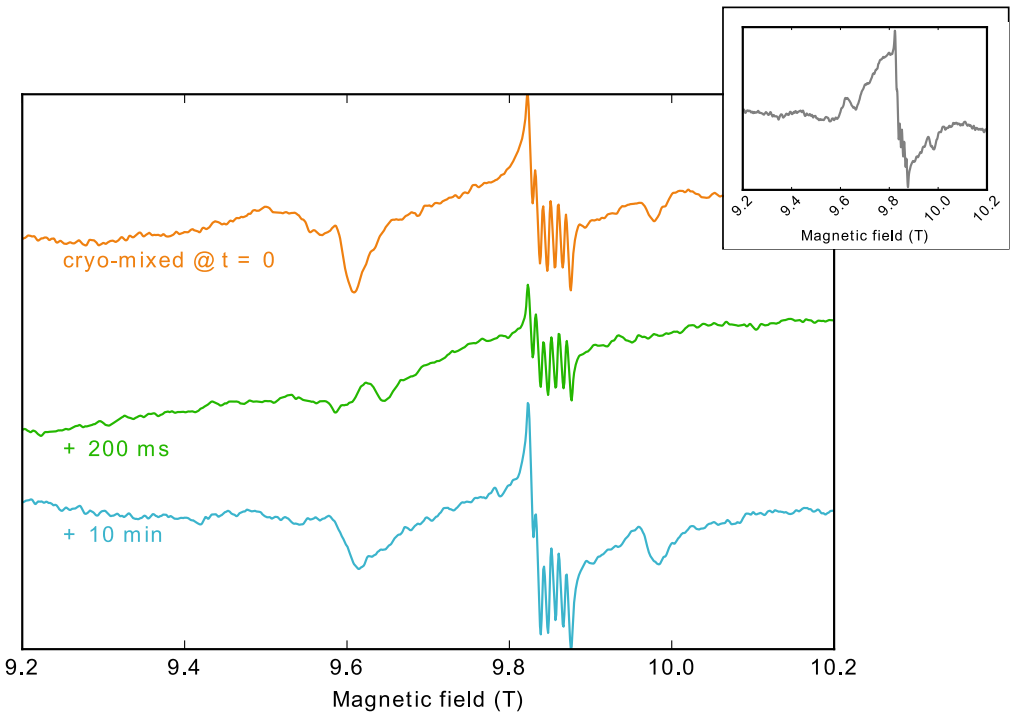

Figure 5.5: 275 GHz cw EPR spectra of the cryo-mixed sample, i.e., T1D SLAC reduced with excess sodium dithionite, mixed with oxygen-saturated buffer with the "sub-zero mixing" technique. Orange: $t = 0$ (no laser); green: after two consecutive laser pulses of 100 ms each; light blue: after laser irradiation for 10 minutes. Each laser pulse promotes the oxidation of T1D SLAC caused by the warming of the enzyme solution. The inset shows the signal of a solution of oxygen-saturated buffer only, without T1D SLAC.

Although not one of the motivations of the present research, for the first time the 275 GHz cw EPR spectrum of the intermediate of T1D SLAC is recorded here, with a structure clearly distinct from that of the reoxidized form. Such spectrum thus provides the basis for the description of the electronic structure of the intermediate, in combination with the data from 9.5 GHz EPR and possibly more frequencies. As mentioned earlier in this Chapter, the intermediate of T1D SLAC is currently understood as a triplet state originating from the coupling of the $S = {}^1/_2$ spin of T2Cu^{2+} and of the $S = {}^1/_2$ spin of a tyrosyl radical [85] [86] [88]. Given the high-spin nature of a triplet, a multi-frequency EPR approach is particularly advantageous in unraveling certain parameters of the spin Hamiltonian describing the system, especially regarding the dipolar

coupling and the exchange interaction of the two spins of the triplet. Further details on high-spin systems in EPR are provided in Chapter 1 of this thesis.

A comparison of the spectra obtained from the RT-mixed sample and the cryo-mixed sample indicates that the latter at $t = 0$ (orange spectrum in Figure 5.5) shows a signal, which does not correspond to the intermediate spectrum obtained from the RT-mixed sample (red spectrum in Figure 5.4), but rather it appears to contain at least a considerable amount of reoxidized T1D SLAC form. This is highlighted by the difference (shown in purple in Figure 5.6) between the spectrum of the hand-mixed sample at $t = 0$ and the spectrum of the RT-mixed sample after 10 min laser irradiation to room temperature, corresponding to the reoxidized T1D SLAC form. Although far from being conclusive, the difference clearly lacks the spectral feature of the reoxidized T1D SLAC form, while at the same time containing peaks roughly in the same field region as those of the spectrum of the T1D SLAC intermediate. These observations point to the fact that the sub-zero mixing of T1D SLAC with oxygen-saturated buffer has not resulted in a true $t = 0$ sample, but rather to one where part of the reaction has already taken place, and thus a mixture of the reoxidized form and of the intermediate are visible.

The main reason to the largely unsuccessful sub-zero mixing of the sample reported in this Chapter is to be found in the temperature at which the mixing took place, namely -30 °C instead of -50 ÷ -60 °C, like described in Chapter 4. The temperature of -30 °C might not have been low enough for the system under study not to react. For instance, supposing that at -30 °C the evolution of the intermediate of T1D SLAC (which occurs within milliseconds at room temperature) is slowed down by three or even four orders of magnitude, still the sample spends a long enough time at that temperature for the reaction to have progressed considerably. Indeed, the sample spends about 30 minutes at -30 °C during the procedure to mix the components and pack the capillaries, plus a couple of minutes more prior to the application of the laser-induced T-jumps.

Considering the non-standard conditions under which the sub-zero mixing of the enzyme was performed in this Chapter, and taking into account the successful results obtained with T-Cycle EPR on the sub-second time scale thanks to the sub-zero mixing in Chapter 4, there is reason to believe such method can be applicable to the study of the reoxidation of T1D SLAC and, more in general, to any kinetic investigation involving enzymes. Further experiments are required to prove the applicability of sub-zero mixing on such systems.

In conclusion, in this Chapter the applicability of Temperature-Cycle EPR was tested on the reoxidation of T1D SLAC, an oxygen-reducing enzyme. In particular, the experiments reported

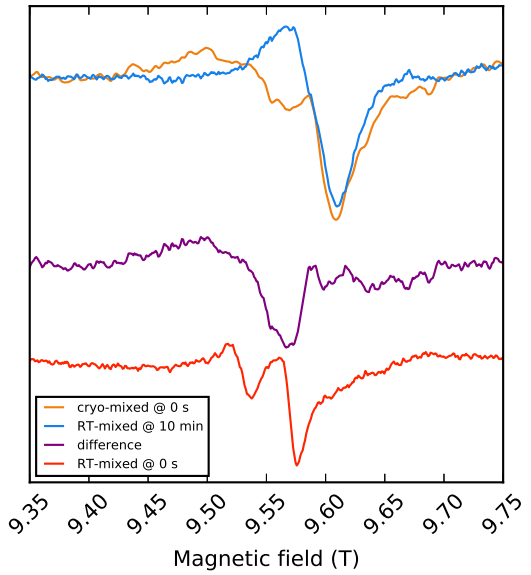

Figure 5.6: Comparison of the 275 GHz cw EPR spectra of the RT-mixed sample at $t = 0$ (red) and 10 minutes (blue) from Figure 5.5, with the spectrum of the cryo-mixed sample at $t = 0$ (orange) Figure 5.4 B. The blue spectrum is subtracted from the orange one (purple spectrum) after baseline correction and normalization by the manganese signal.

here suggest that T1D SLAC does not suffer from exposure to cyclic temperature changes, either cryostat-controlled or laser-induced (which took place continuously over days), thus making such system eligible for T-Cycle EPR. Moreover, the preliminary results of the application of laser-induced T-jumps of the order of hundreds of milliseconds point out that sub-second T-Cycle EPR on enzyme systems is feasible, provided a well-defined background-free sample at $t = 0$ is available. Achieving such starting sample by means of the sub-zero mixing technique described in Chapter 4 will require further testing, but there is no evidence that such goal is out of reach. By and large, there is indication that the way is paved towards investigations on enzyme kinetics with T-Cycle EPR.