Enzymatic reduction of oxygen by small laccase. A rapid freeze-quench EPR study

Nami, F.

Chapter 3

Rapid-freeze-quench EPR up to 275 GHz

Rapid freeze-quench (RFQ) in combination with electron-paramagnetic-resonance (EPR) spectroscopy at X-band is a proven technique to trap and characterize paramagnetic intermediates involved in enzymatic reactions on the time scale of milliseconds. We report on the systematic extension of the RFQ/EPR methodology to higher microwave frequencies. Samples for EPR are prepared by sucking the suspension of RFQ particles into the EPR tube. In this way the particles are efficiently packed into the capillaries with inner diameters down to 150 μm that fit into the single-mode cavities for high-frequency EPR. We demonstrate that one RFQ sample for each point in time suffices for EPR experiments at multiple microwave frequencies. We validate the application of the method to biological samples using the reaction of binding azide to myoglobin, combining RFQ with EPR at 9, 94 and 275 GHz.

3.1 Introduction

Mechanistic studies of enzymatic reactions require detection and characterization of reaction intermediates. Such reactions often involve oxidation/reduction steps that result in the formation of paramagnetic intermediates[123]. The investigation of such intermediates by electron-paramagnetic-resonance (EPR) provides insight into the nature of the paramagnetic center, its conversion during the reaction and its interaction with the chemical environment. Enzymatic intermediates are usually short-lived, and have to be trapped to allow their study by EPR. A proven technique to trap the intermediates on the timescale of milliseconds is rapid freeze-quench (RFQ), which involves freezing a reacting mixture at successive points in time. Commercial RFQ equipment is available, which controls the mixing of the reactants and the subsequent reaction time. The reaction time is set by the length of the reactor and the flow rate. At the end of the reactor the reaction is quenched, commonly by spraying the reaction mixture into cold isopentane. This results in small particles floating in the isopentane, which have to be packed into the EPR tube.

As yet, the RFQ technique, introduced in 1961[4], is mostly combined with EPR spectroscopy at X-band[5,6,7,8,9]. Mechanistic studies would benefit significantly from an extension to other microwave frequencies. Higher microwave frequencies in combination with higher magnetic fields provide enhanced resolution, which facilitates the assignment of EPR spectra for organic radicals with small g-anisotropy and for multiple paramagnetic species. Higher microwave frequencies enable a multi-frequency approach, which is helpful for systems with spin $S > ½$. An additional advantage of high-frequency spectrometers equipped with a single-mode cavity is that sub-microliter sample volumes are sufficient,

which means that little RFQ sample is needed. However, scaling up RFQ/EPR to higher microwave frequencies, the subject of this report, is not trivial.

The first combination of RFQ and high-frequency EPR, at 190 and 285 GHz[10], concerned experiments in transmission mode without a microwave cavity, which largely simplifies the loading procedure of the EPR sample, be it at the cost of sensitivity. While EPR tubes with an inner diameter of 3 mm are being used at 9 GHz, single-mode cavities at higher microwave frequencies can only accommodate capillaries with significantly smaller diameters, e.g. outer diameter of 840 μm in our 94 GHz setup and 250 μm in our 275 GHz setup. This makes sample packing and loading in the traditional way impractical if not impossible. In addition, it is difficult to handle such small capillaries at low temperature, which is essential for RFQ samples.

The first attempt to pack the RFQ particles into small EPR capillaries (i.d. 700 μm) for measurements at 94 GHz was reported by Schünemann et al.[10] in the study of the reaction of cytochrome P450cam with peroxy acids. The mixing was done using a commercial RFQ setup and the reaction mixture was sprayed into isopentane in a glass funnel connected to the EPR capillary. The frozen particles were directly collected from the funnel into the EPR capillary using a steel rod. In this approach, most of the protein sample could not be packed into the EPR capillary. Manzerova et al.[11] employed RFQ/EPR at 130 GHz to study the exchange-coupled thiyl radical–cob(II)alamin system in ribonucleotide reductase. A commercial RFQ setup for mixing was combined with spraying the reaction mixture on rotating copper wheels partially immersed in liquid nitrogen. The collection of the frozen particles into EPR capillaries (i.d.

500 μm) was done using a home-built packing stage immersed in liquid nitrogen. Kaufmann et al.[12] developed a RFQ setup for HF-EPR analysis based on a microfluidic mixer and a cold rotating metal disk for freezing the sample. The sample collection was done by poking the 94 GHz capillary connected to a polypropylene tip into the frozen particles. The performance of the RFQ setup was evaluated using the reduction of the nitroxide radical TEMPOL with sodium dithionite, and the samples were analyzed at 94 GHz. Pievo et al.[13] presented a custom-designed commercial microfluidic RFQ setup in combination with home-built sample collectors suitable for 9, 34 and 94 GHz tubes. To prepare the RFQ samples in the capillaries (i.d. 700 μm) for 94 GHz, the reaction mixture was sprayed into cold isopentane in a small cylinder and the frozen particles were first finely ground using a home-built grinder tool. Then the sample powder was collected by gently patting the capillary on the bottom of the sample collector. The performance of the RFQ setup was evaluated only at X-band, using the binding reaction of azide to myoglobin and samples prepared in EPR tubes of different diameter. To date, no data for RFQ samples analyzed at multiple microwave frequencies has been reported.

In this paper, we demonstrate a systematic approach to the combination of the rapid-freeze-quench technique with EPR spectroscopy at microwave frequencies up to 275 GHz. Recently, we reported a new and efficient way to collect freeze-quench particles for X-band EPR[14]. The method is based on sucking the particle suspension into the EPR tube. Here we extend this method to capillaries with an inner diameter of only 150 μm. In addition, one RFQ sample is found to be sufficient to perform EPR experiments at 9, at 94, and at 275 GHz. We validate the application of

this procedure to biological samples using the reaction of binding azide to myoglobin.

3.2 Materials and methods

Myoglobin (Mb) from equine heart and sodium azide (NaN_3) were purchased from Sigma-Aldrich. Both Mb and NaN_3 were dissolved in sodium phosphate buffer, 100 mM, pH 7.8. A stock solution of 20 mM manganese chloride ($MnCl_2$) in milli-Q water was used.

The concentration of the myoglobin solutions was determined spectrophotometrically using an extinction coefficient $\varepsilon_{505} = 9.7\ mM^{-1}cm^{1}$.

3.2.1 RFQ device

The RFQ experiments were performed using an Update Instrument System 1000 Chemical/Freeze Quench Apparatus with a Model 1019 syringe ram and a Model 715 ram controller. The reactant syringes are connected to the mixer via the coupling hoses in a T-shaped inlet arrangement. The mixing starts when the two reagents meet and is considered to be complete before the reaction mixture reaches the reactor. The reaction takes place in the reactor, which connects the outlet of the mixer to the nozzle. The reaction time is tuned by variation of the length of the reactor and of the velocity of the syringe plungers.

We use isopentane as cryogenic medium at the temperature of –135 to –140 °C. Pre-cooled nitrogen gas is used to maintain the temperature of the isopentane bath during the sample preparation. The temperature of the bath is monitored with a thermometer equipped with a Type K thermocouple (OMEGAETTE, model HH308). The isopentane bath is continuously stirred to ensure a homogeneous temperature. A glass tube (Duran, 12 ml) is filled with cold isopentane and equilibrated in the

isopentane bath. The opening of the tube is covered to minimize warming up. Once the isopentane in the glass tube reaches the desired temperature, we start the sample preparation. The reagents are rapidly mixed and the reaction mixture is sprayed through the nozzle into the isopentane in the glass tube, which causes instantaneous quenching of the reaction.

3.2.2 EPR measurements

The 9.5 GHz spectra were recorded on an ELEXSYS E680 spectrometer (Bruker BioSpin GmbH) equipped with a He-flow ESR900 Cryostat (Oxford Instruments) and a standard TE102 cavity.

The 94.1 GHz spectra were recorded on a Bruker ELEXSYS E680 spectrometer equipped with a He-flow CF935 Cryostat (Oxford Instruments) and a home-built probe head with a single-mode cavity specially designed for continuous-wave (cw) measurements.

The 275.7 GHz spectra were recorded on a home-built spectrometer[15] equipped with a He-flow CF935 Cryostat (Oxford Instruments) and a home-built probe head with a single-mode cavity specially designed for cw measurements[16].

All 94 and 275 GHz spectra were recorded after temperature stabilization of approximately two hours and the actual temperature of the cavity was verified by a calibrated Cernox resistor (Lake Shore Cryotronics) attached to it.

3.3 Results

3.3.1 Preparation and loading of RFQ samples

Our method to prepare RFQ/EPR samples is similar for EPR tubes of different inner diameter down to 150 μm. This method concerns sucking

the RFQ particle suspension into an EPR tube with a filter inside. The transport liquid (isopentane or liquid nitrogen) exits through the filter while the frozen particles are trapped in front of the filter. Depending on the size of the EPR tube for the measurement at a particular microwave frequency, the type of filter and the way of handling is different.

RFQ/EPR sample for 9 GHz

A polypropylene disk with a thickness of 1.57 mm and a pore size of 35 μm (purchased from Scientific Commodities, Inc.) is used as a filter. The filter with a diameter of 2.8 mm is punched out of a polypropylene plate and pushed down to the bottom of the EPR tube. The 3 mm (i.d.) EPR tube is open from both sides and one side is tapered to keep the filter in position. Once the filter is properly placed, the filter side of the EPR tube is connected to a water aspirator through latex tubing. The EPR tube assembly is precooled in cold isopentane at the temperature of dry ice. The glass tube containing the freeze-quench sample is quickly transferred from the isopentane bath to a polystyrene box filled with dry ice. The open end of the EPR tube is inserted in the glass tube while the filter side is kept on dry ice to avoid warming up the sample. The particle suspension is sucked into the EPR tube by the aspirator. Once the particles are inside the tube, they are more tightly packed using a stainless-steel rod. The aspirator is stopped when all liquid isopentane is sucked out of the EPR tube. The EPR tube is disconnected from the aspirator by cutting the latex tube using a precooled cutter. The sample is kept in liquid nitrogen until further use.

RFQ/EPR sample for 94 GHz

The RFQ sample in the X-band tube is transferred from liquid nitrogen to a polystyrene box filled with dry ice. The space in the box above the dry ice is flushed with a flow of pre-cooled nitrogen gas to prevent warming up of the sample. A small amount of RFQ particles is transferred from the X-band EPR tube to a home-built steel mortar containing cold isopentane (at −80 °C), and subsequently ground with a pre-cooled steel pounder. A suitable capillary (i.d. 600 μm) contains a dental-paper-point filter inside and at one of the two open ends is connected to a 50-ml syringe. The fine-ground RFQ particle suspension is sucked into the capillary and trapped in front of the filter while the transport liquid, isopentane, goes through the filter. The larger the underpressure created with the syringe, the better the packing of the RFQ particles. The sucking is stopped when 6 to 8 mm of the tube is filled with the particles. The sucking procedure takes one to two minutes. The capillary is disconnected from the syringe by cutting the capillary using a precooled cutter.

RFQ/EPR sample for 275 GHz

The preparation of the RFQ sample for the EPR measurement at 275 GHz is similar to that for 94 GHz. In this case the inner diameter of the capillary is only 150 μm and the sample volume is only 20 nl. To avoid warming up of the sample, all the manipulations are performed at lower temperature, in a polystyrene box filled with liquid nitrogen instead of dry ice. Isopentane is also replaced by liquid nitrogen as the transport liquid and silica-gel powder is used as a filter.

Sample loading at 94 and 275 GHz

Our 94 and 275 GHz spectrometers are both equipped with home-built probe heads (figure 3.1 A), which carry horizontally positioned single-mode cavities. A loading stage (figure 3.1B) is connected to the probe head, which accommodates a specially designed metal block (figure 3.1C) whose centre is in line with the cavity. The metal block consists of four parts, a base part and three upper parts, each of which has a groove in the middle. Before loading, the probe head and loading stage are cooled to −80℃. The cold metal block that carries the capillary filled with the RFQ sample is placed on the loading stage. The whole construction is continuously under a flow of pre-cooled nitrogen gas.

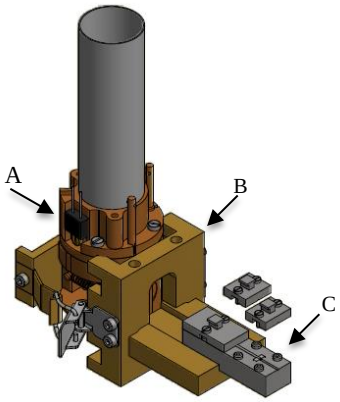

Figure 3.1. Drawing of the specially designed tools for loading the capillaries with the RFQ/EPR sample into the resonator. A) The home-built probe head, which carries a horizontally positioned single-mode cavity. B) The loading stage, which is clamped to the probe head during loading of the sample. C) The metal block with a groove in the middle, which consist of four parts, a base part and three upper parts. The filled capillaries for the 94 and 275 GHz experiments are stored in the metal block until loading.

Removing the upper parts of the metal block one by one, the capillary is smoothly pushed into the cavity while the part of the capillary with the sample remains covered.

3.3.2 The myoglobin-azide reaction

We consider the test reaction of binding azide to myoglobin to validate the procedure of sample preparation and demonstrate RFQ/EPR at 9, 94 and 275 GHz with one set of RFQ samples, i.e., one RFQ sample for each point in time.

Myoglobin at neutral pH exists in the high-spin (HS) Fe(III) S = 5/2) form. The HS form, in which one of the axial positions of iron is occupied by a water molecule, exhibits an EPR spectrum with a sharp signal at g = 6 and a small signal at g = 2. The weakly bound water is readily replaced by exogenous ligands such as azide. This ligation change leads to the low-spin (LS) Fe(III) S = 1/2) configuration, which gives rise to a different EPR spectrum with a rhombic g-tensor around g = 2 (Appendix, figure 3A.1).

The freeze-quench samples were prepared by mixing 4.8 mM myoglobin with 60 mM sodium azide, both in 100 mM sodium phosphate buffer at pH 7.8. The azide solution contained 50 μM $MnCl_2$ as an internal standard for high-frequency measurements. The experiments were performed with the 2 ml syringes in 1:1 mixing mode and the flow rate of 2.7 ml s^{-1}. For each sample, 150 μl of mixed solution was sprayed into isopentane. The reaction was allowed to proceed for different times between 2.6 and 8.8 ms before being quenched. The time was calculated from the ram velocity, the length of the reactor and the mixer volume.

Azide binding to myoglobin shows up in the EPR spectra at 9, 94 and 275 GHz as a decrease of the HS iron signal with increasing reaction time. The spectra at three reaction times are represented in figure 3.2.

The EPR spectra at the same reaction time but different microwave frequencies correspond to one and the same RFQ sample. At 9 GHz the spectra contain in addition the LS iron of the reaction product (figure 3.1.a ESI). At 94 and 275 GHz the LS iron signals are very broad but the sharp six line spectrum of Mn(II) clearly shows up (figure 3.1.b and c ESI). Both the LS iron signal at 9 GHz and the Mn(II) signals at 94 and 275 GHz have been used to normalize the HS iron signals.

In the presence of a 10-fold excess of azide, the binding reaction exhibits pseudo-first order kinetics

$$\ln \frac{[HS]_t}{[HS]_0} = -k_{app} \times t \qquad (1)$$

$$k_{app} = k \times [N_3^-] \qquad (2)$$

where k is the rate constant and $[HS]$ and $[N_3^-]$ are the concentration of HS myoglobin and azide, respectively. The subscripts t and 0 corresponds to the reaction times.

The magnitude of the HS iron signal, which is proportional to $[HS]$, depends on the packing density as well as on the amount of the RFQ particles packed into the EPR tube. These vary from sample to sample, which means that the kinetic analysis of the EPR data requires normalization.

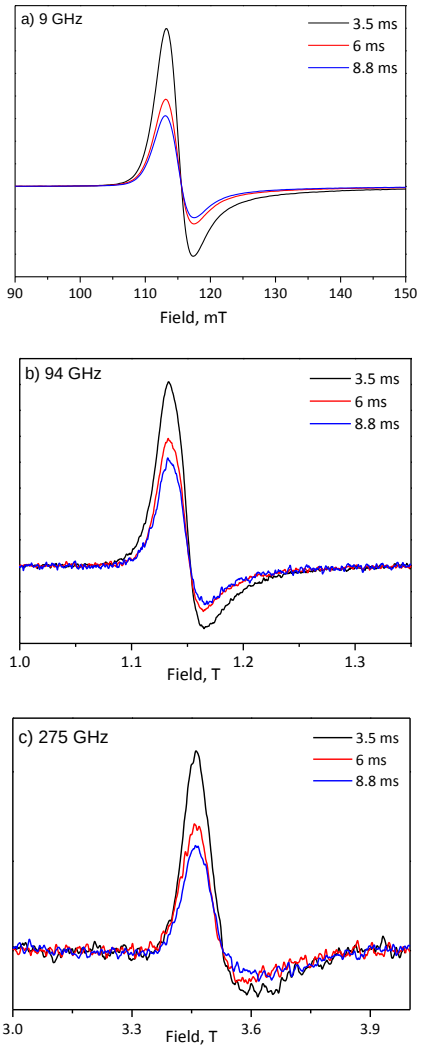

Figure 3.2. The EPR spectra of HS iron for different reaction times recorded at different microwave frequencies. All the spectra are normalized (see the text). EPR measurements: (a) microwave frequency 9.5 GHz, T = 20 K, microwave power 0.16 mW and modulation amplitude 0.5 mT (b) microwave frequency 94.1 GHz, T = 20 K, microwave power 50 μW and modulation amplitude 1.1 mT (c) microwave frequency 275.7 GHz, T = 10 K, microwave power 1.8 μW and modulation amplitude 1.3 mT.

We define $[HS]_t$ / $[HS]_0$ as $Y(t)$. At 94 GHz and 275 GHz, we obtain $Y(t)$ from the ratio of the normalized EPR intensities, at times t and 0:

$$Y(t) = \frac{[(I_{HS})_N]_t}{[(I_{HS})_N]_0} \tag{3}$$

where

$$(I_{HS})_N = \frac{I_{HS}}{I_{Mn(II)}} \tag{4}$$

Here subscript N refers to the normalized intensities and I_{HS} and $I_{Mn(II)}$ refer to the peak to peak intensities of the corresponding EPR signals centered at 1.15 T and at 3.37 T (the 5th line of Mn(II) counting from low field) for 94 GHz and at 3.50 T and at 9.86 T for 275 GHz.

According to Pievo *et al.*[13], at 9 GHz the ratio $Y(t)$ can be written as

$$Y(t) = \frac{R_t}{R_t + \lambda} \tag{5}$$

where

$$R_t = \frac{(I_{HS})_t}{(I_{LS})_t} \tag{6}$$

and

$$\lambda = \frac{(I_{HS})_0}{(I_{LS})_\infty} \tag{7}$$

Here I_{HS} and I_{LS} refer to the intensities of the corresponding EPR signals centered at 116 mT and at 240 mT.

Figure 3.3 shows the values of ln $Y(t)$ as a function of the calculated reaction time. For the data at 94 and 275 GHz, the points represent

average values of measurements on up to four samples. All experimental points of the measurements at 275 GHz are given in figure 3A.2.

The rate constants (k) derived from the slopes of the linear fits and the concentration of azide, and the freezing times from the intercepts are given in Table 1. The linear fits to the 9 and 275 GHz data coincide, while the fit to the 94 GHz data has a slightly higher slope.

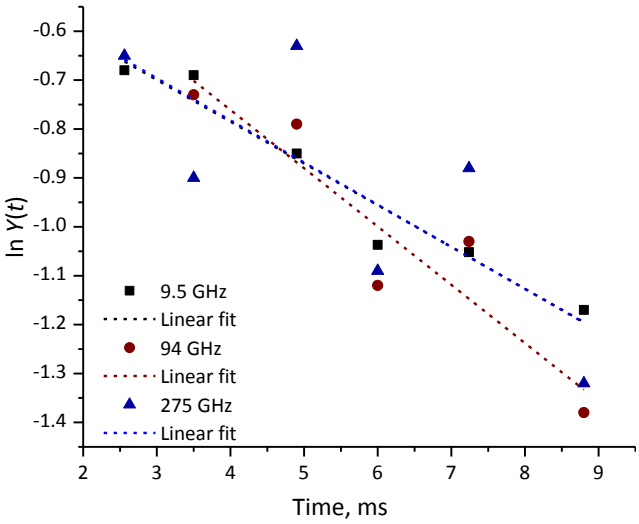

Figure 3.3. Semilogarithmic plot of $Y(t)$ as a function of the reaction time. The lines correspond to linear fits of the experimental data at different microwave frequencies. Those for 9 GHz and 275 GHz virtually coincide.

Table 1. Rate constants (k) and freezing times derived from the EPR measurements of the RFQ samples at different microwave frequencies for the reaction of myoglobin and azide. The k is calculated from k_{app} according to eq. 2 with azide concentration of 30 mM.

EPR frequency (GHz)	k ($\times 10^{-3}$) (M^{-1} s^{-1})	Freezing time (ms)
9	2.9±0.3	5.2±0.7
94	4.0 ± 0.9	2.4 ± 1.4
275	2.9 ± 1.1	5.1 ± 2.5
all frequencies	3.1 ± 0.5	4.2 ± 0.9

3.4 Discussion

We have developed a systematic approach to combine the rapid freeze-quench technique with multi-frequency EPR spectroscopy up to 275 GHz. For each point in time, one RFQ sample suffices for EPR experiments at all frequencies. The method to prepare the RFQ/EPR samples is the same for EPR tubes of different inner diameter. The method concerns sucking a suspension of RFQ particles into the EPR tube with a filter inside, which allows the transport liquid (isopentane or liquid nitrogen) to exit while the frozen particles are trapped inside the tube. Recently, we found that the sucking method is an efficient and easy way to collect freeze-quench particles from isopentane and pack them into a three millimeter (i.d.) X-band EPR tube[14]. Here we have extended the sucking method to narrow capillaries with an inner diameter down to 150 μm. Small amounts of

RFQ particles are suspended in cold isopentane or liquid nitrogen from which such capillaries are easily filled.

We have demonstrated the combination of RFQ with EPR at 9, 94 and 275 for biological samples using the reaction of binding azide to myoglobin. Figure 3.3 shows that the data concerning the conversion of Fe(III) from HS to LS obtained from the same samples at the three microwave frequencies are mutually consistent. The combined analysis of all data yields a rate constant for azide binding to myoglobin at pH 7.8 of $3.1 \pm 0.5 \times 10^3\ M^{-1}s^{-1}$ and a freezing time of 4.2 ± 0.9 ms, both in good agreement with reported values [11,17,18].

Noteworthy though is the spread in the data in figure 3.3, in particular the increase of the spread going to higher frequencies. When the data at X-band are analyzed separately, the standard deviation (RMSD) of $\ln Y(t)$ amounts to 0.05, for 94 GHz to 0.10, and for 275 GHz to 0.16. Moreover, the points at 94 and 275 GHz in figure 3.3 are averages of up to four measurements on EPR samples prepared from the same RFQ sample (see Appendix, figure 3A.2). For example, the point at 6 ms corresponds to -1.12 ± 0.01 for 94 GHz, and to -1.09 ± 0.23 for 275 GHz (for 9 GHz there is only one measurement corresponding to the whole RFQ sample).

In order to check the reproducibility of the EPR measurements at 275 GHz, we performed several experiments on unreacted ("t = 0") HS Fe(III) myoglobin samples. The results show that the EPR measurements cannot be the origin of the scatter of the data. We have to conclude that the variation derives from the RFQ samples. Neither the collection efficiency nor the packing factor can play a role, since all intensities are normalized. Most likely, the variation derives from inhomogeneity of the RFQ

samples, which is in line with the observation that the spread increases with increasing microwave frequency. With increasing microwave frequency, the volume of the EPR sample decreases. At X-band the EPR signal is averaged over 150 µl of RFQ sample. At 94 GHz and at 275 GHz the EPR signals concern sub-samples of 2µl and 20 nl, respectively. The smaller the sample the more the inhomogeneity shows up.

Since the same RFQ sample was planned to be measured by EPR from 9 to 275 GHz, we have deliberately chosen a relatively high concentration of myoglobin, 2.4 mM, and consequently a high concentration of azide, 30 mM, to ensure pseudo-first-order kinetics. The apparent rate constant, k_{app}, of about 90 s^{-1} corresponds to a half-life time of about 8 ms for the binding reaction. This means that in less than 16 ms more than 75% of the reagent has reacted. Taking into account the freezing time of about 4.2 ms, the binding reaction was followed between 7 to 13 ms. As can be seen in figure 3.2, the signal to noise ratio of the spectra corresponding to the longest reaction time are still high enough that the reaction could have been followed for a longer time. Following such a fast reaction with a difference of 1 ms between each point in time by RFQ/EPR is rather challenging. Inhomogeneity of the samples most likely results from a distribution in mixing and/or freezing times. If mixing, rather than freezing, is the primary source of the spread in the data, as pointed out by Manzerova *et al.*[11], the problem could possibly be reduced by using a more efficient mixer.

We have not found any report in the literature of data obtained from analyzing RFQ samples at multiple frequencies to compare with our data. The two reported RFQ devices designed for multi-frequency EPR have been both evaluated at X-band[11,13].

The use of one RFQ sample for EPR experiments at different microwave frequencies has clear advantages compared with the preparation of a new RFQ sample for each microwave frequency. Not only is much less material required, it also ensures that the EPR experiments at different frequencies concern the same stage of the reaction. This is important for the characterization of intermediates and for kinetic studies. For example, enzymes like oxygenases, oxidases, hydrogenases and nitrogenases make use of molecular oxygen, hydrogen and nitrogen as substrate or co-substrate. Due to the gaseous nature of these substrates, their concentration is difficult to control, but significantly influences the kinetics. It is then difficult to make sure that the same time for different RFQ samples corresponds to the same stage of the reaction.

We have used a standard commercial RFQ device, but our preparation procedure can well be combined with a microfluidic device. Coupling the standard RFQ setup with only one high-frequency EPR experiment is inefficient. While the amount of RFQ sample required for each high-frequency experiment is less than 2 μl, the amount of reactants required for the standard RFQ setup is more than 60 μl for each point in time. A large amount of sample is wasted. A microfluidic device has the advantage of a smaller dead volume, and the combination with the sucking procedure would reduce the material consumption for the RFQ experiments. This is the more so because one RFQ sample can be used for EPR at multiple frequencies. Moreover, the sucking method has the advantage that the RFQ particles are taken into capillaries with the cold transport liquid (isopentane or liquid nitrogen), which reduces the risk of heating the sample during packing.

3.5 Conclusion

We have shown that RFQ/EPR experiments at different microwave frequencies, up to 275 GHz, can be performed on the basis of one RFQ sample for each point in time. Sucking the RFQ particles into EPR tubes or capillaries is an economic and user-friendly way to prepare samples for EPR. We used the classical reaction of binding azide to myoglobin to study the feasibility of the method, and we found mutually consistent results at different frequencies. We observed an increase in the scatter of data going to higher microwave frequencies. Because the measurements at different microwave frequencies concern samples prepared from one and the same RFQ sample, we can trace back this observation to inhomogeneity of the RFQ sample. Such inhomogeneity becomes more prominent in EPR at higher frequencies, because the sub-samples that we study become smaller.

For the reaction of myoglobin with azide, measuring the EPR at different frequencies does not add new information. However, for the characterization of paramagnetic intermediates of enzymatic reactions, the use of more than one microwave frequency might well be essential. This applies, for example, to our mechanistic study of the reduction of oxygen by a multicopper oxidases, SLAC[20], which is the subject of chapters 4 and 5.

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Appendix

Rapid-freeze-quench EPR up to 275 GHz

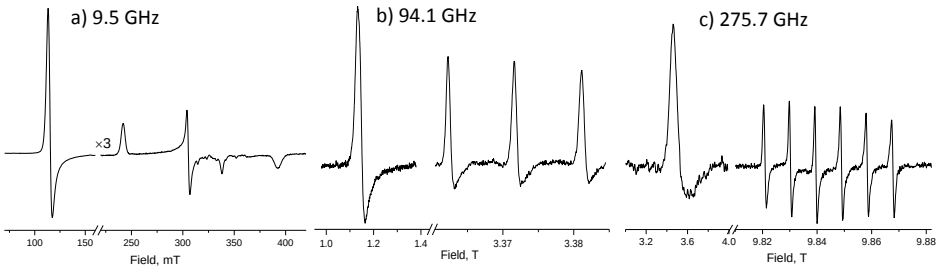

Figure 3A.1. Full cw EPR spectra of the RFQ myoglobin/azide/Mn(II) sample at the reaction time of 3.5 ms for 9, 94 and 275 GHz. At 9.5 GHz the spectrum contains in addition to the HS iron signal the LS iron signal of the reaction product. At 94 and 275 GHz, the LS iron signals are very broad but the sharp line spectrum of Mn(II) clearly shows up. At 94 GHz only three lines of Mn(II) signal were measured. EPR measurements: (a) T= 20 K, modulation amplitude/frequency 0.5 mT/100 kHz and microwave power of 0.16 mW,. (b) The Fe(III) myoglobin part: T = 20 K, modulation amplitude/frequency of 1.1 mT/6 kHz and microwave power of 50 μW. The Mn(II) part: T = 90 K, modulation amplitude/frequency of 0.2 mT/6 kHz and microwave power of 2.0 μW. (c) The Fe(III) myoglobin part: T = 10 K, modulation amplitude/frequency of 1.3 mT/1.7 kHz and microwave power of 1.8 μW. The Mn(II) part: T = 10 K, modulation amplitude/frequency of 0.3 mT/1.7 kHz and microwave power of 0.8 μW.

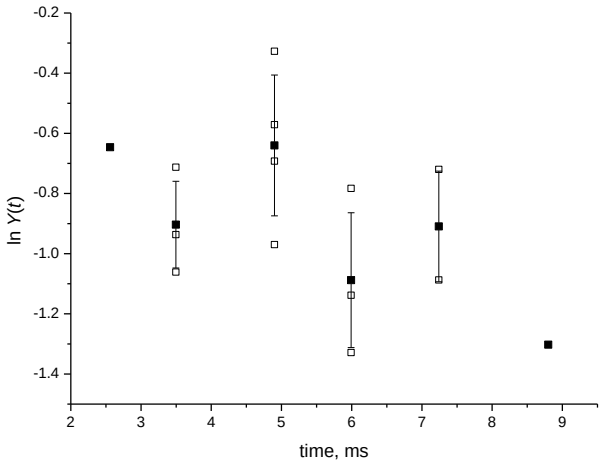

Figure 3A.2. Semilogarithmic plot of $Y(t)$ including all measurements at 275 GHz as a function of the reaction time for the binding of azide to myoglobin. The measured and average points are presented by open and solid squares, respectively.