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

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Summary

A wealth of information on chemically reactive systems can be derived by kinetic studies. In chemistry, knowledge of reaction rates and intermediates sheds light on the reaction mechanisms and, more in general, on the function of the (bio)chemical system under study in relation to its environment. However, an intrinsic difficulty of kinetic investigations are the time scales on which many chemical systems react, and the even shorter time scales on which their intermediates exist.

Rapid Freeze-Quench (RFQ) is one of the most widely used techniques to investigate chemical kinetics. Given the paramagnetic nature of the intermediates of many reactions, coupling RFQ to Electron Paramagnetic Resonance (EPR) is a desirable goal in order to unravel the structure, mechanism, and function of a range of reactive systems, such as enzymes. In particular, high-frequency EPR (HF-EPR) is the spectroscopic methodology of choice in studying paramagnetic species because it offers high resolution and better definition of the magnetic parameters. On the other hand, collection of RFQ samples for HF-EPR is troublesome since very small capillaries are used as sample holders. In **Chapter 2**, the successful coupling of RFQ to HF-EPR at 275 GHz is described. Sample collection performed with a previously developed method is efficient and reproducible. Furthermore, the approach allows the use of only one single series of RFQ samples, to be employed at any EPR microwave frequency, thus enabling multi-frequency EPR investigations. Important advantages of this approach are the reduced amount of material needed (which is particularly beneficial for biological samples), and the improved consistency of the method.

RFQ poses serious difficulties when it comes to sample packing, amount of material, and reproducibility, so that an alternative approach is highly desirable. **Chapter 3** describes the development of Temperature-Cycle EPR (T-Cycle EPR), a novel high-frequency EPR technique that couples laser-induced T-jumps of the sample to a high-frequency 275 GHz EPR spectrometer, in order to detect short-lived paramagnetic intermediates and kinetics of chemical reactions in aqueous solutions. In T-Cycle EPR, a mixture of reactants – initially at a temperature where

no reaction occurs – is heated in situ by means of an infrared laser pulse, in a homogeneous, reproducible, and controllable manner. This *in-situ* T-jump increases the temperature of the mixture so that the reaction can take place for an arbitrary period of time. The application of a sequence of T-jumps to the mixture thus lets the molecular reaction of interest unfold step by step, and a kinetic study of the chemical system is possible. In Chapter 3, it is first shown that T-Cycle EPR works, by observing a chemical reaction on the time scale of minutes. Secondly, a series of experiments are described to show that reactions at different temperatures can be studied, which makes T-Cycle a flexible and powerful technique for the investigation of (bio)molecular dynamics at temperatures other than room temperature, in contrast to RFQ. Thirdly, a method is described to calibrate the temperature of the sample. Important advantages of T-Cycle EPR are the use of only one sample to carry out a kinetic study, which does not pose limitations on the reproducibility of the sample preparation, and the need for only a small amount of material. Both characteristics are beneficial with respect to other methods that require larger amounts of material and/or multiple samples, such as RFQ.

Chapter 4 discusses the application of T-Cycle EPR on a model reaction that takes place over several hundreds of milliseconds, proving the technique is suitable for the study of many (bio)chemical systems. A new hand-mixing method is described to easily and efficiently mix the reagents at low temperatures without them reacting, which can in principle replace RFQ. Furthermore, quantitative kinetics can be obtained provided an analysis of the sample's temperature profile during the laser pulse is performed, which also yields the effective reaction time per T-jump, and the dependence of the rate constant on temperature. In its current state, T-Cycle EPR offers the advantages of HF-EPR with a time resolution already optimal for a wide range of (bio)chemical systems. Additionally, in studies whose goal is the investigation of the evolution of paramagnetic reaction intermediates rather than quantitative kinetics, the present setup could even be employed in the time regime of tens of milliseconds. Finally, COMSOL simulations provide some insights on future improvements of T-Cycle EPR, in terms of sample volume, cryostat temperature, and material of the sample holder.

A pioneering attempt to apply T-Cycle EPR to the study of an enzymatic system on the sub-second time scale is presented in **Chapter 5**. The system under study is the T1Cu-depleted mutant of Small Laccase (T1D SLAC), an oxygen-reducing enzyme, which oxidizes a number of diverse substrates. T-Cycle EPR experiments at 275 GHz are performed on the reoxidation in the sub-second time regime of fully reduced T1D SLAC, to prove the applicability of the method to an enzymatic system, without making use of RFQ. The experiments reported in Chapter 5 suggest that T1D SLAC does not suffer from exposure to cyclic temperature changes, 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.