Studying enzyme kinetics by dissolution DNP: methodological developments and application to metabolomics.

I - Context of the PhD research project and objectives

The study of cellular metabolism and **enzymatic kinetics is central to the understanding of cellular function**. Indeed, the plasticity of the enzymatic activity allows the cell to survive the modifications of its environment through the regulation of its metabolic pathways, thus allowing to maintain the homeostasis of the cell. Over 500 metabolic diseases associated with enzyme dysfunctions, and affecting many different metabolic pathways are known to date.
Analyzing metabolic pathways and their mutual couplings represents a novel approach to develop strategies to identify new targets or therapeutic molecules. A particularly striking example is the case of multiple sclerosis, where the use of biotin, a co-factor of several key enzymes, leads to clinical improvement [1]. A detailed description of metabolic fluxes and their regulation in the cell is therefore clearly essential to better understand the effects of their alteration. Thus, fluxomics approaches attempt to model the various metabolic pathways by numerical methods in order to try and predict the effects of certain drugs. This type of approach therefore requires the **characterization of enzymatic kinetics under physiological conditions**, a particularly crucial aspect.
We propose to explore and characterize these metabolic kinetics by nuclear magnetic resonance spectroscopy (NMR), a versatile and efficient method widely used both in research and in industry. NMR allows to study the structure and dynamics of molecules. One of the most interesting aspects is the possibility to acquire time-resolved information by NMR, which can be used to study systems that evolve with time, such as chemical reactions, where the evolution of substrates and products can be simultaneously monitored. However, one of the major limitations of NMR is its intrinsic lack of sensitivity, sometimes leading to prohibitive experiment durations. This is extremely detrimental for the study of fast kinetic processes (< 60 s) in real time, therefore often difficult to perform. This lack of sensitivity is due to the weak polarization of nuclear spins at thermal equilibrium in a magnetic field. This polarization (the relative population difference between two spin energy levels) is of the order of 10^{-4} at room temperature, even when using the strongest magnets to date (> 23 T).
Several so-called “hyperpolarization” methods have been developed. Among them, **dynamic nuclear polarization** (or DNP) and in particular **dissolution DNP (or D-DNP)**, that is, its application to solution NMR, is at the heart of this project. **D-DNP experiments can increase the NMR signal intensity by four orders of magnitude** [2]. Since its invention, many instrumental developments have taken place and various applications, mostly to health-related problems, have been proposed. The D-DNP experiment consists of several steps: i) nuclear hyperpolarization at very low temperature (~ 1.2 K) through microwave irradiation of the paramagnetic spin impurities (carried by the so-called "polarizing agent"); ii) transition to the liquid phase (dissolution) by flushing onto the hyperpolarized sample a solvent heated to 180ºC, then; iii) transfer through a fluid path, under helium gas pressure, to an NMR spectrometer and finally; iv) acquisition of NMR spectra on the hyperpolarized spins. This last step is repeated to probe the time evolution of the system under study.
One of the major advantages of D-DNP is the possibility to observe fast *in vitro* enzymatic reactions, at physiological time scales, by injecting the hyperpolarized substrate in a solution containing the enzyme, into the NMR spectrometer. Indeed, the extreme sensitivity of the method typically allows the acquisition of an NMR spectrum every 500 ms - 1s, which therefore sets the time resolution of the sampling of the reaction to particularly short values. This is generally not possible with conventional techniques, especially for ^{13}C observation, which often requires an accumulation of spectra to increase the signal to noise ratio, thus limiting the temporal resolution of the kinetics sampling to values that are typically on the order of a minute.

The present project belongs to an extremely stimulating field of research, namely the study of enzymatic kinetics under "pseudo-physiological" conditions. Also, it extends fundamental and applied studies undertaken in both laboratories.

II - Objectives of the research project

The proposed project aims at developing the D-DNP methodology for the observation, analysis and modeling of the kinetics of enzymatic reactions involved in various fundamental metabolic pathways and perturbed in certain pathological contexts.

The first one concerns the **metabolism of glutamine** in the perspective of a search for treatments against diffuse **large B-cell lymphoma (DLBCL)**. It has been shown that glutamine metabolism is disturbed in these cancer cells due to their high energy requirements, as well as mitochondrial oxidative phosphorylation and glucose metabolism. Glutamine is thus a potential target of choice for new anti-metabolic therapies aimed at depriving tumor cells of their energetic supply [3-6]. The best possible characterization of these metabolic transformations, especially in terms of kinetics, is therefore highly relevant.

DLBCL is a particularly aggressive variety of non-Hodgkin lymphoma and is involved in many therapeutic failures (nearly 40% of patients are resistant to treatment). In order to overcome the limitations of conventional therapies (spindle poison, alkylating agents, ...), new **anti-metabolic strategies** are currently being studied. Particular attention is given to the study of **L-asparaginase (**commercially known as **Kidrolase)**. This enzyme was initially known for its role in the conversion of asparagine to aspartate, but is also active in the conversion of glutamine to glutamate. Its synergy with other anti-metabolic therapies (such as metformin, a mitochondrial respiration inhibitor) may induce massive apoptosis of DLBCL cells. In addition, NMR studies of intra- and extracellular metabolomes of DLBCL cells have shed light on the way **these anti-metabolic drugs impact on DLBCL metabolomes** [7]. In particular, it is possible to monitor treatment-specific pathways followed by the endo- and exo-metabolomes.

In another but related perspective, glucose metabolism is known to be disturbed in various pathological contexts, in particular tumors, where cell proliferation is accompanied by an increase in glycolysis (**Warburg effect**) but also by an increased solicitation of the **pentose phosphate pathway (PPP),** whose oxidative stage represents the main source of reducing power in the cell, through the production of NADPH by two enzymes, Glucose-6-Phosphate dehydrogenase and 6-Phosphogluconic dehydrogenase (resp. G6PDH and 6PGDH). The PPP plays a key role in the response to oxidative stress, one of the known factors involved in oncogenesis.

During this project we will therefore continue to develop both the D-DNP methodology for the study of enzymatic kinetics, and its application to the study of key enzymes of these two metabolic pathways.

One of the limitations of D-DNP studies of enzyme kinetics resides in the difficulty to obtain sufficiently reproducible experiments in order to extract parameters from kinetic models in a reliable manner. This is mainly due to the instrumentation set-up used for transferring and mixing the hyperpolarized substrate with the enzyme solution. Modifications of the instrumentation, already in progress in the lab, will allow one to perform both injection (of the hyperpolarized substrate) and mixing (with the enzyme solution) in a controlled way. This new device will be implemented on the D-DNP experiments. Performing series of kinetic experiments with different, and controlled, enzymatic activities will give access to the extraction of kinetic parameters.

Moreover, a second methodological objective aims at the refinement of kinetic models by developing **D-DNP experiments with multiplexed detection,** thereby allowing one to observe several nuclei simultaneously ($^{13}C/^{1}H$, $^{31}P/^{1}H$). This is particularly important in the case of the PPP enzymes G6PDH and 6PGDH, where the latter involve NADP+ as a cofactor. So far, kinetics studies in the lab have been performed in large excess of the co-factor and interpreted as pseudo-first order kinetics. By co-polarizing both the substrate and the co-factor, and using multiplexed NMR, the simultaneous observation of the evolution of substrates and products will be performed and the data will be analyzed in terms of higher order kinetic models.

The developments of the DDNP methodology described above will also be applied to the study of the **mechanism of action of Kidrolase on glutamine and asparagine**. Indeed, although the action of Kidrolase was highlighted by a metabolic profiling of cancer cells, mechanistic and notably kinetic information is still missing to understand and optimize a potential treatment. Several milestones will be set: first, we will validate a method to study the kinetics of the enzyme, which involves the optimization of the sample formulation, the transfer of the hyperpolarized sample to the NMR spectrometer, and the implementation of the acquisition strategies described above, in particular based on a multiplex $^{1}H/^{13}C$ acquisition. Then, we will exploit the acquired data by developing an improved kinetic model, which will be made possible by the new measurables made available. Finally we will interpret these models by explaining in detail the inhibitory

role of Kidrolase in the particular context of its combination with Metformin. It should be emphasized that a better characterization of the action of Kidrolase on glutamine and asparagine would constitute a real added value in the **understanding of its mechanism of action against DLBCL**, and a valuable complement to the analysis of the metabolic profiles performed so far.

Very few laboratories in the world have so far published studies in this field (for a recent overview of techniques, see for example [8-9]). This is the case of the "Structure and dynamics of biomolecules" team of the Laboratoire des biomolécules (LBM, UMR7203) which has a strong experience at the interface between DNP instrumentation and its applications to biological problems (enzymatic kinetics and PPP) [10-11]. On the other hand, the "Bio-Spectroscopies" team of the Laboratoire de chimie et biochimie pharmacologiques et toxicologiques (LCBPT, UMR 8601, Université de Paris) is stongly involved in biology- and health-related problems, and in particular in metabolomics, through the development of dedicated methods for the acquisition of NMR spectra with very high resolution, and the use of D-DNP. The two laboratories propose a complementary combination of knowledge and skills, as well as a novel instrumental platform that allow to investigate the above aspects of cellular metabolism under pathological conditions by hyperpolarized NMR.

III- Interdisciplinarity of the project and implementation method

The research project presented is at the interface between biology, pharmacology and advanced approaches in NMR spectroscopy. In order to be successful, it requires the combination of diverse skills, in the domain of D-DNP techniques as well as regarding the development of metabolomics techniques. The project will thus require the candidate to acquire **knowledge and skills at the interface of biology, chemistry and physics**, such as: glutamine metabolism and cancer markers in cellular metabolism; role and action of L-asparaginase, *in vitro* sample preparation; advanced NMR techniques as well as physical basis of D-DNP and instrumentation of D-DNP (cryogenics, microwave sources, ...)

The candidate will be required to adapt to the many facets of the project, which deals mainly with biological issues but combines NMR spectroscopy and engineering skills: part of the work will aim at improving the instrumentation prototypes, with a particular emphasis on the D-DNP instrumentation system to ensure fast and reproducible transfer and injection of the hyperpolarized substrate, and homogeneous mixtures of the injected and receiving solutions (containing enzymes or enzyme cocktails).

Performing D-DNP experiments on biological samples will require the optimization of the substrate polarization step. This will include special attention to the sample formulation that contains the polarizing stable radical, the glassy agents such as glycerol or DMSO (used to avoid sample crystallization and phase partition between radicals and substrate), and the polarization pulse sequence (NMR pulse sequences, microwave irradiation, …).

In order to achieve the objectives of this project, several implementation steps of increasing complexity will be taken. Multiplex pulse sequences and detection on two nuclei will be implemented to develop "classical" NMR monitoring of enzymatic reactions. These experiments will then be developed in their "hyperpolarized" version by D-DNP of *in vitro* enzymatic reactions. A protocol for the processing and analysis of experimental data adapted to hyperpolarized experiment will also be developed in order to determine models and extract the relevant kinetic parameters for the different enzymes and metabolic pathways studied and described above. Finally, the effect of potential inhibitors of the mentioned enzymes will be studied.

The long-term benefits of this project concern first of all metabolomics, with the possibility of applying it to larger series of samples according to the established method, while varying the possible treatments.

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