FIDA-Based Quantification of Small Molecule Binding to Membrane Proteins in Solution

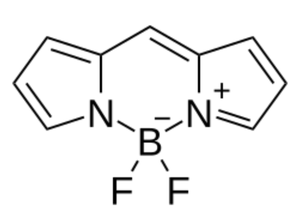
Mehmethan Aris[1], Riccardo Cavalieri[2], Danielle M. Copeman[1], Steven Harborne[2] and Paul G. Crichton[1]

1. Biomedical Research Centre, Norwich Medical School, University of East Anglia, Norwich, United Kingdom
2. Sygnature Discovery Ltd., Tytherington Business Park, Macclesfield, United Kingdom

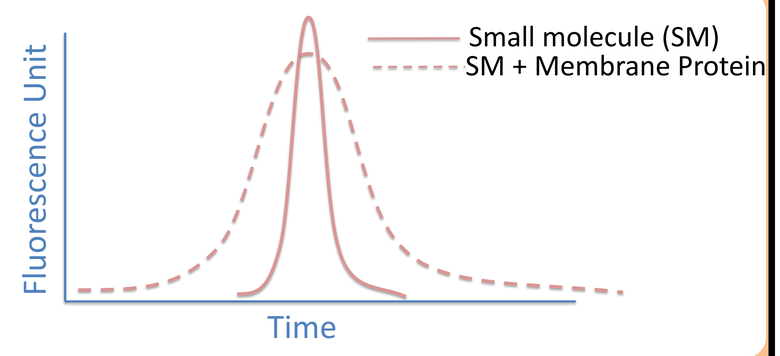
Introduction

Uncoupling Protein 1 (UCP1) is a membrane protein found in the mitochondria of brown adipose tissue (BAT) in mammals and is responsible for heat production through non-shivering thermogenesis. UCP1 dissipates the mitochondrial membrane potential, upon activation by fatty acids, releasing the energy as heat, and is inhibited by purine nucleotides. Human adults have functional BAT, although its abundance decreases with age. Promoting presence of functional BAT and UCP1 has a therapeutic potential and might be important for intervention in the metabolic disorders [1].

We have recently found that human and mouse UCP1 exhibit subtle differences in how they interact with classical UCP1 regulators such as purine nucleotides. We sought to validate this using flow induced dispersion analysis (FIDA). However, It is challenging to track binding or study binding kinetics of membrane protein and small molecules in FIDA applications due to micelle size and limited change in hydrodynamic radius. Alternatively, binding-related intensity change (BRIC) can be used to monitor binding, but fluorescence intensity changes unrelated to binding (e.g., from additives) can complicate interpretation for studying in solution binding kinetics [2]. Instead, we sought to track the size change of fluorescently labelled small molecule as a method to interrogate binding interactions.



BODIPY : 5,5-Difluoro-5H-4λ5-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-4-ylium-5-uide

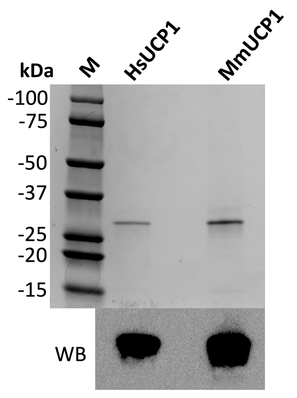


BODIPY labelled small molecule ligand binding assay

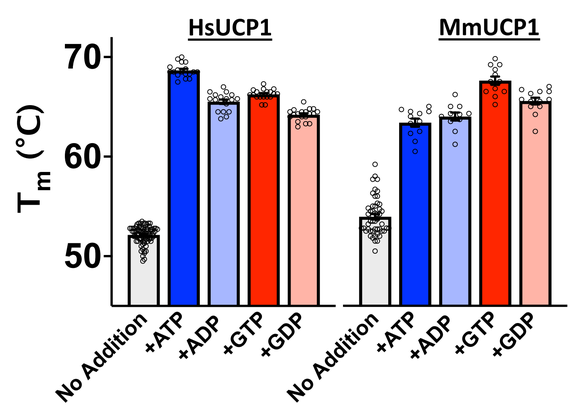
BODIPY labelled small molecule when detected with using 488 fluorescent detector is likely to have a Rh of ~1 nm. The protein of interest is mixed with the BODIPY labelled small molecule either using a CapMix or a PreMix method. As the hydrodynamic radius of BODIPY labelled small molecule is increased over an increasing concentration of analyte (protein), the binding kinetics of the small molecule with a fluorescent adduct can be studied.

1. Species-Dependent Differences in UCP1 Inhibition by Adenosine- and Guanosine-Based Nucleotides

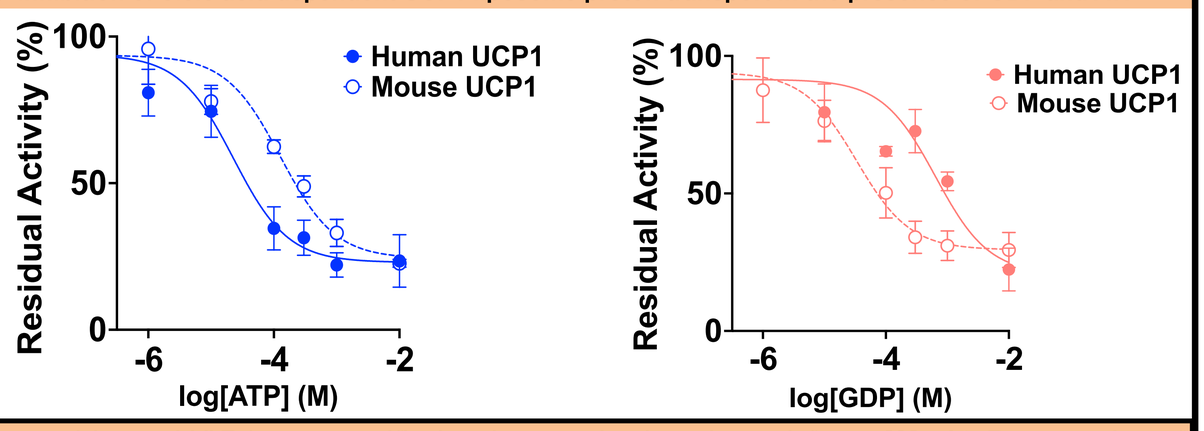
A. Coomassie-stained gel and a western blot showing the homogeneity of UCP1 samples



B. Melting temperatures of Human and Mouse UCP1 in the presence of –di and –tri purine nucleotides



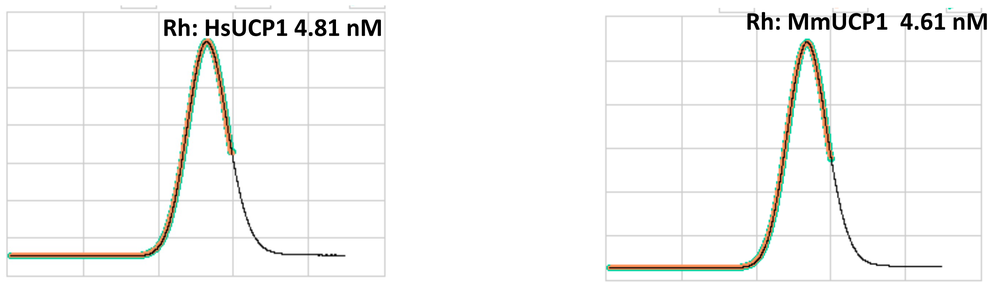
C. Inhibition of oleic acid dependent UCP1 liposome proton transport in the presence of GDP and ATP



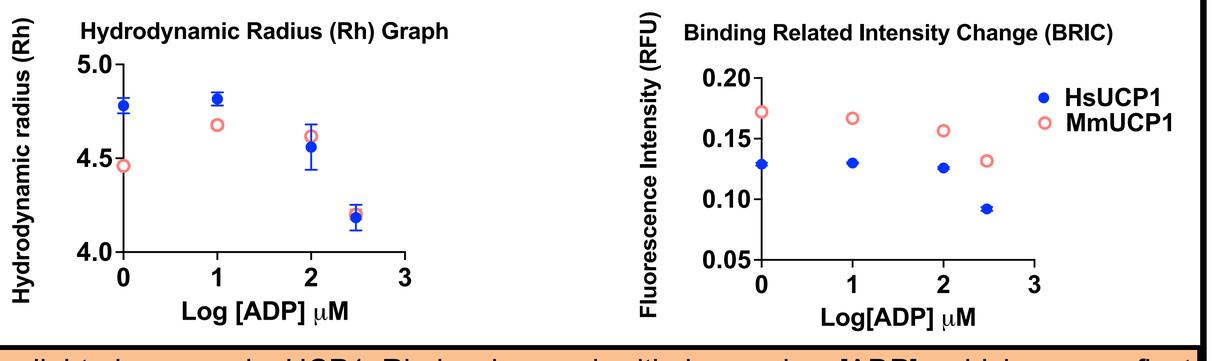
Recombinant UCP1 was purified using methods adapted from [3]. A) Bar graphs of UCP1 orthologs showing their relative melting temperature (Tm, °C) extracted from the peak of derivative fluorescence in the presence or absence of purine nucleotides in the denaturation profiles. Recombinant sheep UCP1 (no addition), n = 73; recombinant sheep UCP1 (+ATP, +GTP), n = 20; native sheep UCP1 (no addition), n = 60; native sheep UCP1 (+ATP, +GTP), n = 8; error bars represent mean ± SEM. B) Coomassie blue and western blot imaging of purified UCP1 orthologs C) Residual activity (normalised to maximal OA-stimulated activity) of human and mouse UCP1 in the presence of differing ATP (blue) and GDP (orange) concentrations (1-10000 µM).

2. Limited Detectability of Nucleotide Binding to UCP1 by FIDA Using UCP1 as the Indicator

A. FIDA traces of HsUCP1 and MmUCP1 without any ligand, obtained using UV detector



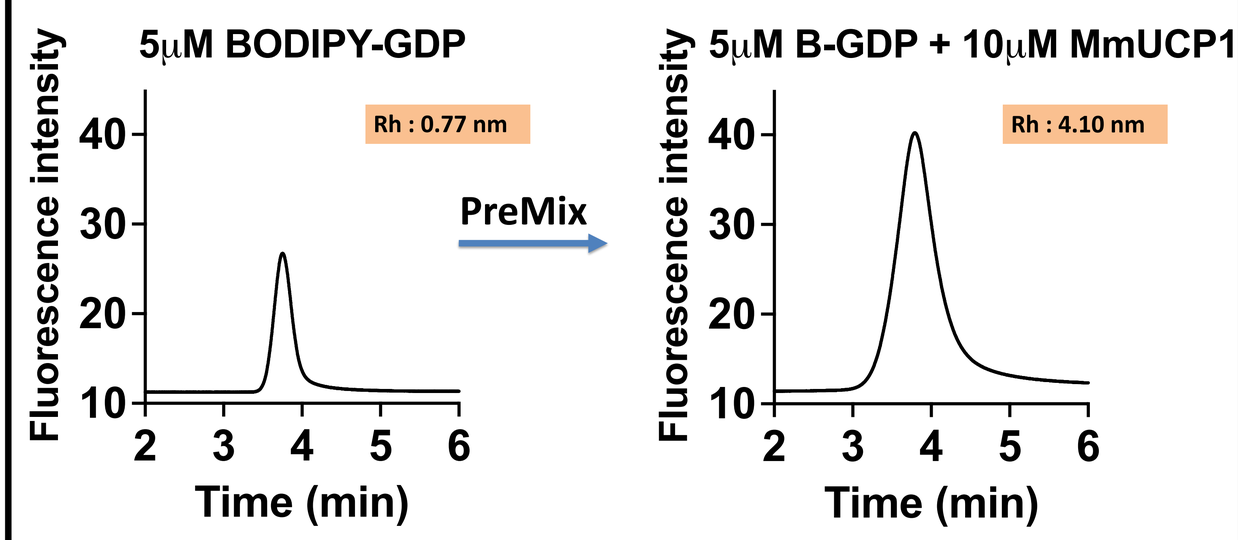
B. Change in the hydrodynamic radius (Rh) and the BRI as a function of small molecule concentration



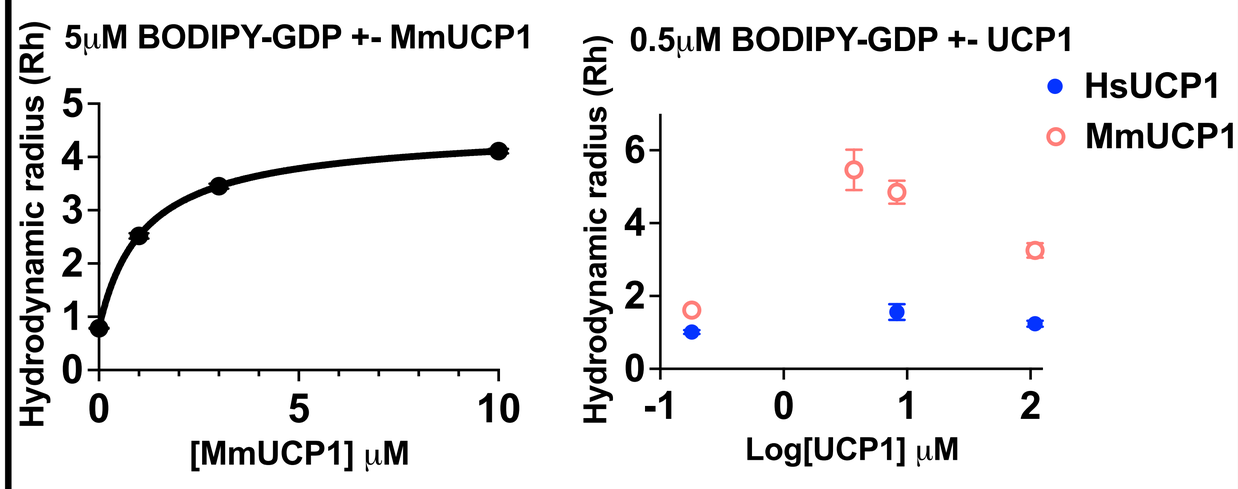
A slight decrease in UCP1 Rh is observed with increasing [ADP], which may reflect conformational changes or a non-specific effect; similarly small decrease in fluorescence intensity (plotted as BRIC) is also observed. Error bars represent mean ± SEM.

3. Using a Small Molecule as the Indicator Enables More Reliable Tracking of Binding Kinetics

A. Example traces of 5 µM BODIPY-GDP and 5 µM BODIPY-GDP + 10 µM MmUCP1 using a PreMix method



B. Change in the Rh of BODIPY-GDP as function of protein concentration



A) Raw FIDA traces of 5 µM BODIPY-GDP (1 specie fit) and +10 µM MmUCP1 (2 species fit) with PreMix method. B) Hydrodynamic radius graph of 5 µM BODIPY-GDP with 1, 3, and 10 µM MmUCP1, data obtained using PreMix Method. Traces were recorded using FIDA 488 Fluorescent detector. 10mM TRIS (pH 7.5), 50mM NaCl, 0.025% Lauryl Maltose Neopentyl Glycol (LMNG) + 18:1 Cardiolipin was used as an assay buffer. Error bars represent mean ± SEM.

Summary

- Adenosine nucleotides bind human UCP1 more tightly than guanosine nucleotides, as opposed to mouse UCP1. ATP and GDP shows the greatest differences between human and mouse orthologs.
- It is challenging to track binding when the ligand bound complex doesn't have significant increase in the Rh compared to unbound.
- BRIC might be indicative of the binding. However, it may not be possible to track binding kinetics and calculate dissociation constant (Kd) reliably.
- It may be possible to track changes in the hydrodynamic radius of a small molecule by labelling the small molecule with a fluorophore adduct to study solution-phase binding kinetics.

References: **[1]** Nedergaard *et al*. (2010) *cell metabolism* 11.4, 268-272; **[2]** Pedersen et al. (2019). Methods in Molecular Biology 1972, 129–142. **[3]** Lee et al. (2015). PNAS 112, 6973–6978.