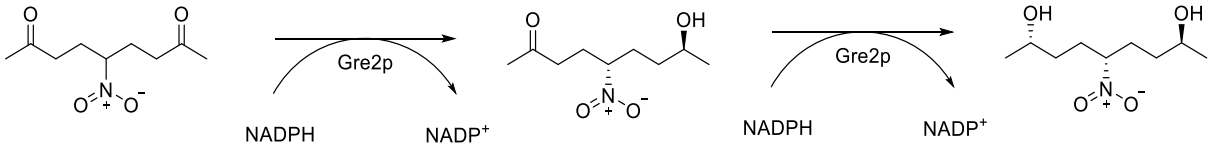
STANDARD OPERATING PROCEDURE (ITC#2_MIM_Gre2p)					
IBG-1 KIT Karlsruher Institut für Technologie		**Institute for Biological Interfaces** IBG-1 | Biomolecular Micro- and Nanostructures			
Short Title	ITC-MIM Gre2p	**Page**	1	**of**	6
Title	Gre2p multiple injection kinetics measurements using the ITC				
Version	1.0		**Created on**	26.04.21	
Safety instructions	The MicroCal PEAQ-ITC uses methanol and a 10% DECON 90 solution for washing of the cell and syringe. Methanol has the following hazards: H225, H301, H311, H331, H370.  DECON 90 has the following hazards: H290, H315, H319.  By following the instructions in this SOP you confirm that you have checked the SDS-sheet of the involved chemicals (also reagents of your specific enzyme system) and will protect yourself accordingly when working with these substances. Also, you confirm that you will follow standard lab safety procedure to protect yourself, others, and the environment.		**Related SOP**	SOP_ITC#1_General SOP_ITC#3_(r)SIM_Gre2p	
			Category	PROTOCOL	
			Purpose	To provide instructions on how to measure kinetics of Gre2p's reaction with NDK as substrate and NADPH as cofactor using ITC.	
			Note	If you are not familiar with the ITC in general, read SOP_ITC#1_General first	
Autor	Felix Ott, Gudrun Gygli				



Reaction scheme of the observed reduction of 5-nitrononane-2,8-dione (NDK) to the corresponding hydroxyketone and possible further reaction to the corresponding diol catalyzed by Gre2p. The cofactor NADPH get oxidized to NADP⁺ in the process.

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1) Introduction

Enzyme kinetics can be measured using ITC multiple injections experiments (ITC-MIM) and ITC (recurrent) single injection experiments (ITC-(r)SIM). ITC-MIM follow the enzyme reaction at each injection and should observe a stabilization of the baseline after injection below the initial value. That way, K_m can be determined. For k_{cat} determination, the enthalpy of the reaction, $\Delta H_{reaction}$, must be obtained with ITC-(r)SIM experiments. In an ITC-MIM experiment, the reactant is titrated to the enzyme to establish a stepwise increase in reactant concentrations, mimicking the classical enzyme kinetics experiment. At each titration step, the energy produced by the enzyme reaction causes a stable shift of the signal, resulting in descending steps. This shift is directly proportional to the rate of the reaction, thereby enabling the calculation of K_m.

2) Preparation of stock solutions

- 100 mM NDK in buffer
  - Store at 4 °C
- 100 mM NADPH in buffer
  - Store frozen and keep dark; freeze again in between measurements
- Gre2p stock (1 – 1.5 mM)
  - Store at – 80 °C; when thawed once, store on ice

3) Sample preparation and loading

Before every measurement, the sample solutions have to be prepared freshly by diluting/mixing the stock solutions.

Cell: 100 – 150 nM Gre2p, 5 mM NADPH

- 198 µL buffer + 2 µL Gre2p stock
- Invert multiple times
- 5 µL of the prepared 1:100 dilution + 25 µL NADPH stock + 470 µL buffer
- Invert multiple times and insert into cell prior to measurement

Syringe: 50 mM NDK

- Mix 40 µL NDK stock with 40 µL buffer in PCR tube
- put tube in loading position

4) Experimental parameters

For the procedure of the actual ITC experiment, check the ITC SOP general. The used parameters are listed in Table 1.

Table 1: ITC parameters used

Parameter	Value
Temperature (°C)	25
Reference Power (µcal/s)	10
FeedBack	High
Stir Speed (rpm)	500
Initial Delay (s)	60
# of injections	19
Volume (µL)	2
Duration (s)	4
Spacing (s)	180

5) Troubleshooting

If the enzyme concentration is chosen too large, this can result in “non-stable”, upwards-tilting plateaus after each injection (Figure 1). Also, make sure to perform appropriate controls: it can happen that the heats of dilution of the ligand(s) “fake” an enzymatic reaction (Figure 2). An example of a successful and good quality measurement is shown in Figure 3.

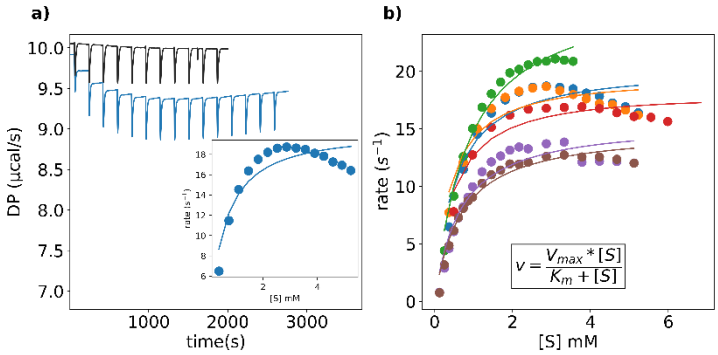

Figure 1: Impact of the enzyme concentration on the kinetics for the conversion of NADPH and NDK by Gre2p in KP_i buffer: blue and orange replicates: 150 nM Gre2p; other replicates: 75 nM Gre2p. a) representative data of the raw heat of the titrations of NDK to NADPH and Gre2p in the cell (blue line), from which isotherms are integrated (inset, dots) and K_m is fitted using the standard Michaelis-Menten model (inset, lines). The heat of dilution of the control (titrating NDK to Gre2p and NADPH in the cell, black) is negligible compared to the heat of the reaction, b) integrated isotherms (dots) and fits for K_m and k_{cat} (lines) of six replicates in blue, orange, green, red, purple and brown. DP stands for “Differential Power”.

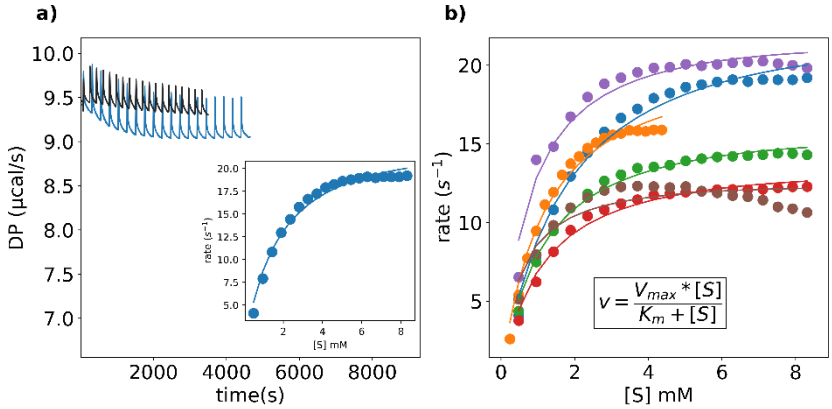

Figure 2: Kinetic parameters for the reaction of Gre2p with its cofactor NADPH and NDK titrating both NADPH and NDK to Gre2p and stirring at 200 rpm in the ITC. a) representative data of the raw heat of the titrations of NDK to NADPH and Gre2p in the cell (blue line), from which isotherms are integrated (inset, dots) and K_m is fitted using the standard Michaelis-Menten model (inset, lines). The heat of dilution of the control (titrating NDK to Gre2p and NADPH in the cell, black) is not negligible compared to the heat of the reaction, b) integrated isotherms (dots) and fits for K_m and k_{cat} (lines) of six replicates in blue, orange, green, red, purple and brown. Note that the data are of poor quality due to the titration of both NADPH and NDK to Gre2p and the too short spacing between injections.

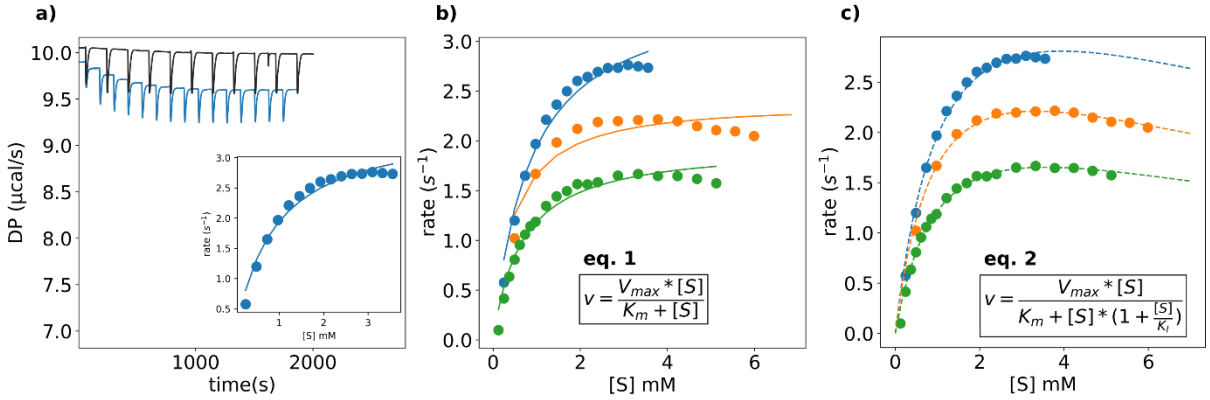

Figure 3: Kinetics for Gre2p from ITC indicate that NADPH is indeed limiting in spectrophotometric assays and that classical Michaelis-Menten model does not represent the data well. Three replicates in blue, orange and green are shown, fitted with different kinetic models in b) and c). a) representative data of the raw heat of the titrations of NDK to NADPH and Gre2p in the cell (blue line), from which isotherms are integrated (inset, dots) and K_m is fitted using the standard Michaelis-Menten model (inset, lines). The heat of dilution of the control (titrating NDK to Gre2p and NADPH in the cell, black) is negligible compared to the heat of the reaction. b) integrated isotherms (dots) and fits for K_m and k_{cat} (lines), note that the standard Michaelis-Menten model does not represent the data well, and that k_{cat} calculated using $\Delta H_{reaction}$ obtained from single injection experiments varies by ± 0.8 s^{-1} between replicates. c) integrated isotherms (dots) and fits for K_m and k_{cat} (lines) with a model assuming substrate inhibition.

6) Enzyme and assay information table

Table 2: Enzyme and assay information summarized following the STRENDA Guidelines as much as possible.

Data	**Value**
Name of reaction catalyst	**G**enes de **r**espuesta a **e**stres (stress-response gene) (Gre2p)
EC number	1.1.1.283
NCBI Taxonomy ID of organism of origin	1294304
GenBank Sequence ID	AJT71311.1
Artificial modification	C-terminal hexahistidine-tag
Storage conditions	
Enzyme purity	Apparently homogeneous by SDS-PAGE
Storage temperature	-80 °C, flash frozen
pH	7.5, measured at 25 °C
Buffer	Depending on the sample in 100 mM KPi, 1xPBS or 100 mM HEPES buffer
Enzyme concentration (of frozen stock)	Depending on the sample in buffer 1.1 to 1.5 mM
Samples are thawed	On ice or at room temperature
Assay conditions	
Substrate purity	NADPH: IWT Reagents with a purity of 99.6 % NDK was synthesized as described here: https://dx.doi.org/10.14272/reaction/SA-FUHFF-UHFFFADPSC-DPTOJTZVUD-UHFFFADPSC-NUHFF-NUHFF-NUHFF-ZZZ HK was synthesized as described here: https://dx.doi.org/10.14272/reaction/SA-FUHFF-UHFFFADPSC-RUKZNVUNYW-UHFFFADPSC-NUHFF-NCEOX-NUHFF-ZZZ
Measured Reaction	Gre2p+NADPH + NDK -> Gre2p + NADP+ + NDK-alcohol, see page 1 of this SOP
Assay pH	7.5, measured at 25 °C for the respective Buffer
Buffer	Depending on the sample in 100 mM KPi, 1xPBS or 100 mM HEPES buffer
Substrate concentration range	5mM NADPH, NDK: 0.3 - 8.0 mM, depending on the individual experiment
Enzyme concentration	Depending on the individual sample in buffer between 100 - 150 nM initially in the cell
Activity and Methodology	
Directly Measured using ITC	