Method Article

A real-time $^{31}$P-NMR-based approach for the assessment of glycerol kinase catalyzed monophosphorylations

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ABSTRACT

Phosphorous-NMR is scarcely employed to evaluate enzyme kinetics of kinase driven monophosphorylations, despite of being a powerful and reliable tool to undoubtedly detect the actual phosphoryl transfer to the targeted substrate. Another advantage is that an external supplementation source of the NMR active isotope is not required, since $^{31}$P is highly abundant in nature. Glycerol kinase (GlpK) from E. coli is an exemplary ATP-dependent kinase/phosphotransferase model to illustrate the value and usefulness of a $^{31}$P-NMR-based approach to assess the enzymatically driven monophosphorylation of glycerol. Moreover, the described approach offers an alternative to the indirect coupled glycerol kinase enzyme assays. Herein, we provided a real time $^{31}$P-NMR-based method customized for the direct assessment of the glycerol kinase enzyme activity.

- Real-time detection for phosphoryl group dynamics in the GlpK driven reaction
- Direct assessment of product formation (glycerol-monophosphate)
- Parallel determination of cosubstrate (ATP) consumption and coproduct (ADP) generation
- Method validation was performed via $^{31}$P-NMR for each phosphorylated molecule involved in the reaction in order to assist in the molecular assignments

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| Name and reference of original method: | [1] RM Werner, A Johnson, $^{31}$P NMR of the pyruvate kinase reaction: An undergraduate experiment in enzyme kinetics, Biochem Mol Biol Educ, 45 (2017) 509–514, doi:10.1002/bmb.21079. [2] JA Walker, JD Friesen, SJ Peters, MA Jones, JA Friesen, Development of a new and reliable assay for choline kinase using $^{31}$P NMR, Heliyon, 5 (2019) e02585, doi:10.1016/j.heliyon.2019.e02585. |
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Method details

Background

To establish the proposed $^{31}$P-NMR-based method and to provide an in-depth illustration of our approach we selected the glycerol kinase enzyme (GlpK) from *Escherichia coli*, which is a widely characterized kinase/phosphotransferase system [3–8]. We performed the reaction without a coupled ATP regeneration system, since the aim was to analyze solely the behavior of the phosphoryl group transfer performed by GlpK.

Glycerol kinase (EC: 2.7.1.30) catalyzes the phosphorylation of glycerol generating glycerol-monophosphate (*sn*-glycerol 3-phosphate) (Fig. 1) [3]. It exclusively uses ATP as phosphor donor and Mg$^{2+}$ is essential for the catalytic activity [3–5]. Substrate affinity for glycerol and ATP is 1.3 μM and 4 mM, respectively [3–5]. The optimum pH is 9.8 and the optimal reaction temperature is 37 °C [3–5]. In addition to the optimum reaction conditions, the interaction of Mg$^{2+}$ and ATP in aqueous phase have a significant effect on the kinetic behavior. Such interaction depends on the Mg$^{2+}$ to ATP molar ratio and results in the generation of Mg-ATP complexes of various physical and chemical features. Consequently, the freely accessible ATP co-substrate for the enzyme is altered, substantially affecting glycerol kinase kinetics [9]. For instance, it has been shown that glycerol kinase from *Cellulomonas sp.* shows a maximum activity at the optimum Mg$^{2+}$ to ATP molar ratio of (0.12 – 0.3) and exhibits a two-step kinetic behavior as function of ATP at fixed Mg$^{2+}$ concentrations. Such behavior correlates with the formation of various Mg$^{2+}$-ATP complexes that bind to the active site [9]. Thus, for a more comprehensive kinetic characterization of the glycerol kinase under study, Mg$^{2+}$-ATP complexes at different Mg$^{2+}$/ATP molar ratios should be considered.

The most commonly employed method to follow kinase phosphoryl transfer reactions is via enzymatic coupled assays. The classic continuous coupled-enzyme assay accounts ATP depletion via an ADP-dependent/NADH-dependent enzyme cascade [10]. The main disadvantage of such approach is that it indirectly reports both the ATP consumption and the phosphorylation of the target substrate. In addition, it is not possible to ascertain unspecific ATP hydrolysis due to factors different from effective biocatalytic product formation. In contrast, a $^{31}$P-NMR based method is capable to detect directly not only ATP consumption or unspecific hydrolysis, but also, product formation and coproduct generation as well. Such comprehensive detection level confers three control points that correlate among each

![Fig. 1. Glycerol kinase (GlpK) catalyzed reaction for the natural substrate glycerol. The enzyme employs ATP as cosubstrate and Mg$^{2+}$ as cofactor to generate glycerol-monophosphate and ADP upon ATP hydrolysis.](image-url)
other since the GlpK driven monophosphorylation of glycerol is equimolar under optimal reaction conditions.

Herein we report a simple \(^{31}\)P-NMR single-tube experiment enabling the real-time assessment of kinase catalyzed monophosphorylations. Our approach is based on the strategies described in the original methods [1] and [2].

This method facilitates reaction dynamics studies for the compounds containing phosphoryl groups involved in the enzymatic reaction.

This approach allows a deep analysis to follow kinetics behavior. Thus, it is also useful for enzyme kinetics characterization and estimation of the equilibrium constant of the reaction under study.

This strategy also allows to investigate correlations in terms of the Mg\(^{2+}\)/ATP molar ratio, which is a major factor for developing an efficient biocatalytic process using glycerol kinase.

In addition, once the \(^{31}\)P-NMR landscape of the actual reaction has been defined, it can be possible to introduce an ATP regeneration system into the set-up reaction to profile its influence on the kinase reaction under study.

We can foresee that the application of the present method can be extended to explore the activity of candidate kinase/phosphotransferase enzymes toward targeted non-natural substrates.

**Method steps**

**Reagents and standards**

Chemicals used in this work were obtained at the highest purity degree available from Sigma-Aldrich (St. Louis, US).

- Glycerol \((M = 92.09 \, \text{g} \, \text{mol}^{-1}, \text{Cat. No. G5516})\)
- ATP; adenosine 5’-triphosphate disodium salt \((M = 551.14 \, \text{g} \, \text{mol}^{-1}, \text{Cat. No. A2383})\)
- ADP; adenosine 5’-diphosphate disodium salt \((M = 427.2 \, \text{g} \, \text{mol}^{-1}, \text{Cat. No. 1905})\)
- Glycerol-monophosphate sodium salt hydrate \((M = 172.07 \, \text{g} \, \text{mol}^{-1}, \text{Cat. No. 61,668})\)
- Magnesium chloride anhydrous \(\text{MgCl}_2\) \((M = 95.21 \, \text{g} \, \text{mol}^{-1}, \text{Cat. No. M8266})\)
- Sodium phosphate dibasic \(\text{Na}_2\text{HPO}_4\) \((M = 141.96 \, \text{g} \, \text{mol}^{-1}, \text{Cat. No. 71,642})\)
- Glycerokinase (GlpK) from *E. coli* \((0.5 \, \text{U} \, \mu\text{L}^{-1}, \text{Cat. No. G6278})\)

**NMR conditions**

\(^{31}\)P-NMR were performed on a Bruker Avance 500 spectrometer operating at 202.47 MHz. Spectra were recorded using \(D_2\)O as solvent.

To enhance signal resolution for the standards and GlpK model reaction, \(^{31}\)P-NMRs were performed using a total of 128 scans at 298.0 K.

To allow rapid measurements during the time-lapse experiment, \(^{31}\)P-NMRs were performed with a total of 16 scans at 305.7 K. The following time intervals were spectra recorded: 0, 12, 30, 40, 48, 116 min.

\(^{31}\)P-NMR spectra processing was performed by using the TopSpin® 3.6.2. software (Bruker BioSpin GmbH, Rheinstetten; Germany).

\(^{31}\)P-NMR-based assessment and validation of GlpK driven monophosphorylation of glycerol

In order to validate the \(^{31}\)P-NMR-based approach to assess glycerol kinase catalyzed monophosphorylations, we performed a model GlpK enzymatic reaction. We employed 10 mM of glycerol as substrate and 10 mM of ATP as cosubstrate, 2.5 units of glycerol kinase, 10 mM of magnesium chloride as cofactor, 50 mM ammonium carbonate buffer pH=9 as solvent (prepared in 10% \(D_2\)O). Reaction volume, temperature, and incubation time were 0.5 mL, 37 °C, and 1 h, respectively. Since the product ATP, ADP, and glycerol-monophosphate are not volatile, reaction was stopped by heating at 95 °C 10 min. The one-hour end-point reaction was analyzed following the described NMR conditions for the GlpK model reaction. The obtained spectra (Fig. 2A) was compared with the standards (Fig. 2B–2E).
Fig. 2. Experimental validation of the $^{31}$P-NMR-based approach for the assessment of glycerol kinase catalyzed monophosphorylations. A, enzymatic reaction employing 10 mM of glycerol as substrate and 10 mM of ATP as cosubstrate, 2.5 units of glycerol kinase, 10 mM of magnesium chloride as cofactor, 50 mM ammonium carbonate buffer pH = 9 as solvent (prepared in 10% D$_2$O). Reaction volume, temperature, and incubation time were 0.5 mL, 37 °C, and 1 h, respectively. B: free phosphate 10 mM, C: glycerol monophosphate 10 mM, D: ADP 10 mM, E: ATP 10 mM. In order to provide the same chemical environment, all standards were solubilized in ammonium carbonate buffer pH = 9 prepared in 10% D$_2$O, containing magnesium chloride 10 mM.

Standards for the establishment of the $^{31}$P-NMR landscape

In order to provide the same chemical environment and to simulate the reaction conditions, all standards (final concentration 10 mM) were solubilized in ammonium carbonate buffer pH=9 prepared in 10% D$_2$O, containing magnesium chloride 10 mM. Samples were submitted to analysis following the described NMR conditions for the standards. The obtained spectra were employed to validate the GlpK driven monophosphorylation of glycerol.

- Na$_2$HPO$_4$ ($^{31}$P-NMR spectra is provided in Fig. 2B)
- Glycerol-monophosphate ($^{31}$P-NMR spectra is provided in Fig. 2C)
- ADP ($^{31}$P-NMR spectra is provided in Fig. 2D)
- ATP ($^{31}$P-NMR spectra is provided in Fig. 2E)

$^{31}$P-NMR single-tube time-lapse experiment

The enzymatic reaction was performed directly in the NMR tube in a 0.5 mL reaction volume, consisting of 50 mM ammonium carbonate buffer pH=9 (prepared in 10% D$_2$O), and equimolar amounts (10 nmol) of each reactant; substrate (glycerol), cosubstrate (ATP), and cofactor (Mg$^{2+}$). After recording the time 0, the reaction was started by the addition of 2.5 units of enzyme. A representative $^{31}$P-NMR spectra stack showing the single-tube time-lapse progress of the GlpK catalyzed reaction at four different times is provided in Fig. 3.

Data processing of the $^{31}$P-NMR single-tube time-lapse experiment

A detailed example for the processing of the $^{31}$P-NMR integrated spectra obtained at minute 12 for the ATP$\gamma$ phosphoryl group and glycerol monophosphate is provided, employing Fig. 4. and Eqs. (1)-
Fig. 3. Representative $^{31}$P-NMR spectra stack showing the single-tube time-lapse progress of the GlpK catalyzed reaction at four different times. The enzymatic reaction was performed employing equimolar amounts (10 nmol) of each reactant in the reaction; substrate (glycerol), cosubstrate (ATP), and cofactor ($\text{Mg}^{2+}$). After recording the time 0, the reaction was started by the addition of 2.5 units of enzyme.

Table 1
Summary of data for every $^{31}$P-NMR spectra recorded during the single-tube time-lapse experiment. Data were processed using Eqs. (1)–(4), as in the example for the 12 min recorded spectra. $^{31}$P-NMR integrated spectra recorded at times 30–116 min are provided in the supplementary material (Figs. S1–S4).

| Time (min) | $^{1}$ATP | $^{2}$GP | $\text{Fx}^{1}$ATP | $\text{Fx}^{2}$GP | ATP (nmol) | GP (nmol) |
|-----------|-----------|-----------|---------------------|------------------|------------|-----------|
| 0         | 1         | 0         | 1                   | 0                | 50         | 0         |
| 12        | 1         | 2.31      | 0.3021              | 0.6979           | 15.11      | 34.89     |
| 30        | 1         | 4.90      | 0.1695              | 0.8305           | 8.47       | 41.53     |
| 40        | 1         | 4.52      | 0.1812              | 0.8188           | 9.06       | 40.94     |
| 48        | 1         | 4.85      | 0.1709              | 0.8291           | 8.55       | 41.45     |
| 116       | 1         | 3.90      | 0.2041              | 0.7959           | 10.20      | 39.80     |

$^{31}$P-NMR integrated spectra obtained at 30, 40, 48, 116 min can be found in the supplementary material section (Fig. S1–S4). A summary for the whole set of processed data is shown in Table 1. Fig. 5 shows the obtained curve for the ATP cosubstrate and the glycerol-monophosphate product after processing the $^{31}$P-NMR spectra recorded during the single-tube time-lapse experiment. The detection tendency, over time, for the generated product is shown in the $^{31}$P-NMR overlay spectra of the single-tube time-lapse experiment for the product glycerol-monophosphate Fig. 6.

An additional comprehensive example for the processing of the $^{31}$P-NMR integrated spectra obtained at minute 12 for the $^{1}$ATP$^{\gamma}$ phosphoryl group along with the ADP$^{\alpha}$ phosphoryl group (Fig. S5) is provided in the supplementary material section, as well as Table S1 and chart (Fig. S6) summarizing the obtained results.

The $^{31}$P-NMR-based approach enables the detection of all molecules harboring phosphoryl groups involved in the reaction, providing three control points that correlate among each other. We plot the calculated remaining fraction of ATP reported in nmol using the integral values for the product glycerol-monophosphate and for the $\alpha$-phosphoryl group of the coproduct ADP. The resulting regression analysis shows a high correlation (0.9867) between the two calculated values (Fig. S7). The
same correlation behavior was observed when plotting the calculated generated amount in nmol for the product glycerol phosphate and the coproduct ADP (Fig. S8). The correlation analysis is useful to verify our approach based on the assumption that the GlpK driven monophosphorylation of glycerol is equimolar under optimal reaction conditions.

31P-NMR data processing

Assumptions

• Calculations for an equimolar reaction, as it naturally occurs under optimal conditions (see Fig. 1)
• ATPγ integral was set to a relative value of 1 for each separate 31P-NMR spectra recorded during the time-lapse experiment, since it is the phosphoryl group being transferred

Example for data processing, employing the integral values for ATPγ and glycerol-monophosphate at 12 min (Fig. 4).

Eq. (1):

\[ F_{xATP} = \frac{\int ATP\gamma}{\int GP + \int ATP\gamma} \]

Term definitions:

\( F_{xATP} \) = Remaining fraction of the employed ATP in the reaction
\( \int ATP\gamma \) = Integral value of the gamma phosphoryl group of ATP
\( \int GP \) = Integral value of the product glycerol phosphate
Fig. 5. Obtained curve for the ATP cosubstrate and the glycerol-monophosphate product after processing the $^{31}$P-NMR spectra recorded during the single-tube time-lapse experiment. ATP •, glycerol-monophosphate ♦.

Fig. 6. $^{31}$P-NMR overlay spectra of the single-tube time-lapse experiment for the product glycerol-monophosphate. The figure shows the recorded signals for glycerol monophosphate generation at six different times in minutes: 0, blue; 12, black; 30, fuchsia, 40, green; 48, gray; 116, red (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Replacing values from Fig. 4 in Eq. (1):

$$F_{xATP} = \frac{1}{2.31 + 1} = 0.3021$$

Eq. (2):

$$F_{xGP} = 1 - F_{xATP}$$

Term definitions:

$F_{xGP}$ = Fraction of the generated product glycerol-monophosphate

$F_{xATP}$ = Remaining fraction of the employed ATP in the reaction (value calculated in Eq. (1))
Replacing values in Eq. (2):

\[ Fx \ GP = 1 - 0.3021 = 0.6979 \]

Eq. (3):

\[ \text{nmol} \ ATP = FxATP \times \text{nmol} \ ATP \ used \ in \ the \ reaction \]

Term definitions: \(\text{nmol} \ ATP = \) Remaining cosubstrate ATP employed in the reaction in nmol
\(FxATP = \) Remaining fraction of the employed ATP (value calculated in Eq. (1)) \(\text{nmol} \ ATP \ used \ in \ the \ reaction = 50 \ \text{nmol} \)
Replacing values in Eq. (3):

\[ \text{nmol} \ ATP = 0.3021 \times 50 \ \text{nmol} = 15.11 \ \text{nmol} \]

Eq. (4):

\[ \text{nmol} \ GP = FxGP \times \text{nmol} \ ATuP \ used \ in \ the \ reaction \]

Term definitions: \(\text{nmol} \ GP = \) Generated product glycerol-monophosphate in the reaction in nmol
\(FxGP = \) Fraction of the generated product glycerol-monophosphate (value calculated in Eq. (2)) \(\text{nmol} \ ATP \ used \ in \ the \ reaction = 50 \ \text{nmol} \)
Replacing values in Eq. (4):

\[ \text{nmol} \ GP = 0.6979 \times 50 \ \text{nmol} = 34.89 \ \text{nmol} \]

Conclusion

Herein we describe a customized version of a robust method for the \(^{31}\text{P-NMR-based}\) assessment of glycerol kinase catalyzed monophosphorylations. The established \(^{31}\text{P-NMR-based}\) approach is a powerful validated strategy enabling the real-time detection for phosphoryl group dynamics, the direct assessment of product formation, and the parallel determination of cosubstrate consumption and coproduct generation. In addition, this method is also useful to study the influence of \(\text{Mg}^{2+}\text{-ATP}\) complexes during biocatalysis under different complexes scenarios (unsaturated, stoichiometric, over-stoichiometric). The approach illustrated herein is employing glycerol as substrate and glycerol kinase as biocatalyst, for an equimolar reaction. However, the application of the described methodology can be extended to investigate reaction dynamics, kinetic characterization, effect of \(\text{Mg}^{2+}\text{/ATP}\) molar ratio, influence of ATP regeneration systems on kinase activity, and activity assessment of candidate kinase/phosphotransferase systems toward selected non-natural substrates.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi: [10.1016/j.mex.2021.101285](https://doi.org/10.1016/j.mex.2021.101285).
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