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# Phosphate ions and glutaminyl cyclases catalyze the cyclization of glutaminyl residues by facilitating synchronized proton transfers

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ABSTRACT

Phosphate ions and glutaminyl cyclase (QC) both catalyze the formation of pyroglutamate (pE, pGlu) from N-terminal glutamine residues of peptides and proteins. Here, we studied the mechanism of glutamine cyclization using kinetic secondary deuterium and solvent isotope effects. The data suggest that proton transfer(s) are rate determining for the spontaneous reaction, and that phosphate and QC are accelerating the reaction by promoting synchronized proton transfers in a concerted mechanism. Thus, non-enzymatic and enzymatic catalysis of pyroglutamate formation exploit a similar mode of transition-state stabilization.



## 1. Introduction

Pyroglutamic acid (pGlu, 5-oxo-L-proline) is present at the N-terminus of numerous peptides and proteins. The residue is formed by intramolecular cyclization of glutamine or glutamic acid [3,20]. The modification blocks N-terminal degradation by aminopeptidases [15,26] and is thought to mediate receptor binding [16,10,7,23]. With only a few exceptions known [2,25], glutamine represents the precursor of pGlu in virtually all physiologically active peptides and proteins. The cyclization of glutamine is considered as an quasi-irreversible intramolecular acyl transfer reaction. The N-terminal peptide amino group nucleophilically attacks the γ-carbon amide, thereby forming a tetrahedral intermediate ($t_i$). The decomposition of this intermediate proceeds by net 1,3-proton transfer from Nα onto the leaving ammonia, whereby the pGlu lactam ring is formed (Fig. 1A, inset).

In vivo, the cyclization reaction is enzymatically catalyzed by glutaminyl cyclases (QCs) [13]. Interestingly, the cyclization of glutamine – but not of glutamic acid – takes place at significant rates under mild conditions even in the absence of the enzyme catalyst [24]. In this context, some anions such as inorganic phosphate have been described to accelerate the pGlu-formation at neutral pH [11]. The detailed mechanism of the catalytic activity has not been investigated so far.

Here, we have examined the non-enzymatic and enzymatic cyclization of glutamine in water and deuterium oxide ($D_2O$) using kinetic isotope effect studies in order to decipher the rate-limiting steps of the cyclization reaction, and to understand how exactly phosphate and QC act as catalysts. Our results suggest that proton transfers are rate limiting for the spontaneous cyclization.

## 2. Materials and methods

### 2.1. Materials

The QC used for enzymatic conversion of N-terminal glutamine was from *Drosophila melanogaster* [21]. Chemicals were purchased as follows: Glutamate dehydrogenase from Fluka, NADH/H+ and α-ketoglutaric acid from Sigma, deuterium oxide (99.8%) from Roth (Germany). Peptides (QFA-$NH_2$, pEFA-$NH_2$, Q(d5)FA-$NH_2$) were synthesized semi-automatically as C-terminal amides as described previously [22]. All other chemicals used were of analytical or HPLC grade.

### 2.2. HPLC assay for evaluation of spontaneous cyclization

The products pEFA-$NH_2$ or pE(d5)FA-$NH_2$ were separated from their precursors QFA-$NH_2$ and Q(d5)FA-$NH_2$ using RP-HPLC on a reversed phase C18 stationary phase (LiChrospher® C18,

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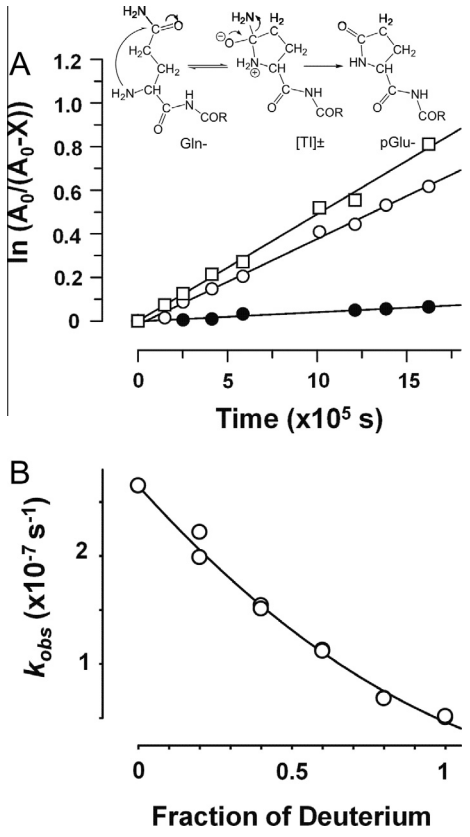


**Fig. 1.** (A) Inset: Mechanism of pyroglutamate formation; main: formation of tripeptide pGlu-Phe-Ala (pEFA) from Gln-Phe-Ala (QFA, circles) or ($d_5$)-Gln-Phe-Ala (d5QFA, squares) at pH (or pD) 7.0. Compared with QFA dissolved in $H_2O$ (open circles; $k_{obs} = 2.7 \pm 0.1 \times 10^{-7}\ s^{-1}$), the rate of cyclization is significantly lower in deuterium oxide (filled circles; $k_{obs} = 0.50 \pm 0.03 \times 10^{-7}\ s^{-1}$). The data were evaluated on the basis of first-order kinetics. (B) Proton inventory analysis for the cyclization reaction. The bowl-shaped curvature is consistent with transfer of 2.2 ± 0.5 protons in the transition state. Data from two independent experiments are plotted, error bars of least-square fits are smaller than the data points.

LiChroCart® 125-4, both Merck, Darmstadt, Germany). The Merck–Hitachi HPLC system was equipped with an L7200 autosampler, L6210 pump and an L4500 diode array detector. Separation to baseline was obtained by applying a linear gradient of 5–30% acetonitrile/0.1% TFA in water/0.1% TFA for 10 min. The concentrations of the products were determined from a standard prepared under assay conditions. The pH dependence of spontaneous pGlu-formation was investigated in a buffer consisting of 0.0125 M Mes, 0.025 M Tris, 0.0125 M acetate, pH 3.0–10.0. This buffer provides a constant ionic strength in the chosen pH range [6]. In all other cases, the ionic strength ($I$) was kept constant by addition of sodium chloride. The concentration of the buffer ions was calculated according to the Henderson–Hasselbalch equation. Alternatively, the pseudo-linear relationship between electric conductance ($G$) and ionic strength, which is valid for low salt concentration ($I \leqslant 0.3$ M) and defined as $\log I = 1.159 + 1.009 \log G$ [12] was used. The peptide concentration was adjusted to 1 mM and reaction temperature was 30 °C, samples of 60 µl were taken at different time points.

### 2.3. β-secondary isotope effects and proton inventory analysis

QFA-$NH_2$ or deuterated isotopolog Q($d_5$)FA-$NH_2$ (0.5 mM) were dissolved in buffer containing 0.05 M MOPS, pH/D 7.0. The determination of pD at the pH meter was based on the equation: pD = meter reading + 0.4 [18]. The reaction temperature was 30 or 60 °C, samples of 60 µl were taken. The proton inventory was recorded by preparing mixtures of 0.05 M MOPS, pH/D 7.0 containing QFA-$NH_2$ such that the mole fractions of $D_2O$ was 0%, 20%, 40%, 60%, 80% or 100%. The enzyme-catalyzed cyclization reaction was analyzed as described previously [22]. Depending on the solvent composition, all ingredients were correspondingly either dissolved in $H_2O$ or $D_2O$. The maximum deviation from a pure deuterium oxide mixture by usage of water-based solution of glutamic dehydrogenase was 2%. All enzymes were preincubated for 60 min with the corresponding buffer. Kinetic data were evaluated using GraFit software (version 5.0.4. for windows, Erithacus software Ltd., Horley, UK). The proton inventory data were evaluated using the equation: $k = 0.026488 * ((1 - n) + (n/(KIE)\hat{}(1/z))\hat{}z)$ [18], in which $k$ is the observed rate constant, $n$ denotes the mole fraction of deuterium, KIE is the maximum isotope effect and $z$ the number of protons transferred in the transition state.

## 3. Results

### 3.1. Glutaminyl cyclization is significantly accelerated by phosphate

The spontaneous cyclization–deamidation of QFA-$NH_2$ was assessed using HPLC-UV. Due to cyclization of QFA-$NH_2$, the N-terminus is rendered more hydrophobic, leading to an increased retention time of pEFA-$NH_2$ (not shown). Formation of reaction product pEFA-$NH_2$ accords to first-order rate law (Fig. 1A, open squares). The half-life of QFA-$NH_2$ is ~29 days at pH 7.0 and 30 °C, thus its cyclization is 10 times slower than that of the model peptide QFRH-$NH_2$, which has been analyzed previously [24]. The reaction rate is virtually pH-independent between pH 4.0 and pH 10.0 (not shown). Similar pH dependence was observed previously with another glutaminyl peptide [24]. Considering that the pH range spans the amine p$K_a$, a potential explanation for the independence of the rate from pH would be that there are two pH-dependent steps in the reaction, which are inversely influenced by pH.

Previous observations pointed to an accelerated cyclization of glutamine in presence of inorganic phosphate [11]. In our hands, increasing the concentration of phosphate resulted in a directly proportional rise of the estimated rate constant of pGlu formation by up to 70 fold (Table 1). Accordingly, the half-life of N-terminal glutamine dropped to 11 h in presence of 0.1 M phosphate at pH 7.0 and 30 °C.

### 3.2. β-secondary and solvent isotope effect studies of glutaminyl cyclization

In order to study the chemical mechanism of phosphate buffer catalysis, we synthesized a deuterated glutaminyl substrate isotopolog [($d_5$)QFA-$NH_2$] containing a deuterated glutaminyl residue. The rate of pGlu-formation from ($d_5$)Gln was significantly higher as suggested by $k_{(H)Gln}/k_{(D)Gln}$ of 0.8 (Fig. 1A, open squares). The observed inverse isotope effect is in accordance with a change of the coordination at the γ-carbonyl carbon in the transition state,

**Table 1**
Kinetic solvent deuterium isotope effects on non-enzymatic and enzymatic conversion of glutamine into pGlu at pH (pD) 7.0 and at 30 °C.

| Catalyst | $k_{obs}$ ($s^{-1}$) (×$10^{-6}$) | | $k_H/k_D$ |
|---|---|---|---|
| | $H_2O$ | $D_2O$ | |
| – | 0.27 ± 0.01 | 0.050 ± 0.003 | 5.4 ± 0.1 |
| PP (0.005 M) | 1.46 ± 0.10 | 0.68 ± 0.38 | 2.2 ± 1.1 |
| PP (0.025 M) | 7.63 ± 0.29 | 3.94 ± 0.28 | 1.9 ± 0.1 |
| PP (0.05 M) | 9.78 ± 0.81 | 6.23 ± 0.25 | 1.6 ± 0.1 |
| PP (0.1 M) | 17.5 ± 1.4 | 12.4 ± 0.5 | 1.4 ± 0.1 |
| QC | 12.5 ± 1 (×$10^6$) | 12.6 ± 0.4 (×$10^6$) | 0.99 ± 0.05 |