Tetrahedron 73 (2017) 4896–4900

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

A trypsin-based bistable switch☆

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ARTICLE INFO

Article history:
Received 6 March 2017
Received in revised form
20 April 2017
Accepted 21 April 2017
Available online 26 April 2017

Keywords:
Complex behavior
Reaction networks
Bistability
Enzymes

ABSTRACT

Recreating some of the emergent behavior seen in biological reaction networks is an important goal in the new field of systems chemistry. One of the classic examples of complex behavior is bistability, which is abundantly used in living organisms for switching between cellular states. Here, we create a bistable switch based on the autocatalytic activation and inhibition of the enzyme trypsin under flow conditions. We investigate the influence of the inhibitor structure, and hence inhibition kinetics, on the properties of the bistable switch.

1. Introduction

All-or-none responses are crucial in cellular processes such as differentiation,[1] cell motility,[2] apoptosis,[3] and cell cycle control.[4,5] This type of response is typically regulated by bistable switches,[6–8] that are able to maintain a stable state A until a trigger switches the system to state B, after which this new state persists even when the trigger is removed, a property called hysteresis. In the past decades, artificial networks have been developed that display bistability, including ones based on DNA circuits,[9] enzymes,[10] inorganic chemistry,[11] and most recently, small organic molecules.[12] In addition, mathematical analyses have indicated that many network motifs can lead to bistability as long as they contain a sufficient degree of nonlinear kinetics.[13,14] We propose to use a basic motif, displayed in Fig. 1A, where an autocatalytic, positive feedback loop in which trypsin (Tr) catalyzes its own formation from its precursor trypsinogen (Tg), is combined with a trypsin inhibitor (Inh). Importantly, trypsinogen displays self-activation due to residual tryptic activity, which ensures a slow, but continuous production of trypsin.[15] This motif is comparable with the one that was described recently, in which thiols were constantly produced through thioester hydrolysis, amplified through native chemical ligation, and inhibited by maleimides.[12] Importantly, bistability is only obtained under out-of-equilibrium conditions in these systems, and to that end we implement a flow reactor.

In this report, we achieve bistability by combining kinetic studies with batch experiments and computational modelling to predict the conditions necessary for bistability in a flow reactor. Previously, we developed this strategy to design a trypsin oscillator based on a different network motif than the one used here.[16] Furthermore, we now synthesize a new, more potent inhibitor of trypsin by modifying a commercially available trypsin inhibitor, and probed the influence of the inhibition kinetics on the properties of the bistable system. As we saw before in the trypsin oscillator,[17] small modifications in molecular structure can have a profound influence on the behavior of complex, out-of-equilibrium networks.

2. Results and discussion

In a reactor, the autocatalytic formation of trypsin is antagonized by the outflow of reaction products.[18] Intuitively, at extremely high flow rates, the outflow of the reactor is similar to its inflow, because trypsinogen is washed out of the reactor before it can be activated. Conversely, one imagines that under batch conditions (no flow) the reaction will proceed until all trypsinogen has been converted into trypsin and thermodynamic equilibrium is reached. Under all circumstances, the addition of inhibitor counteracts the autocatalysis. In a bistable system, a regime exists in which the starting concentration of trypsin determines the final state of the system. At low concentrations of trypsin, outflow and inhibition

☆ The authors congratulate Prof Ben Feringa on being awarded the 2016 Tetrahedron Prize and Nobel Prize in Chemistry.

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http://dx.doi.org/10.1016/j.tet.2017.04.053
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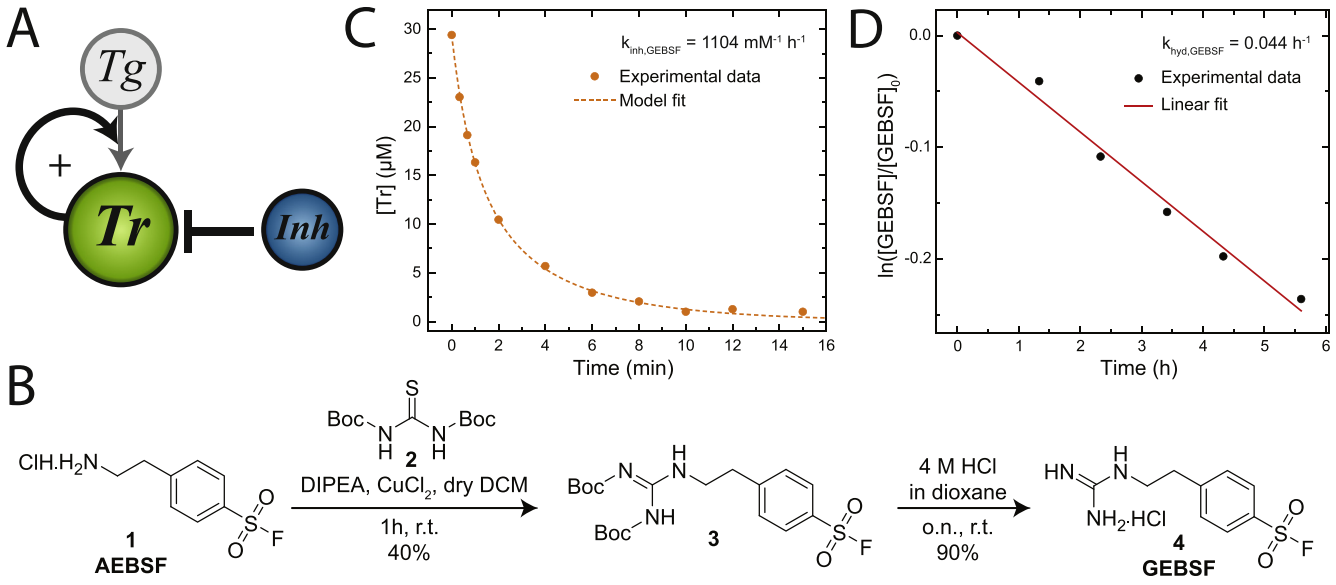

Fig. 1. A) The enzymatic reaction network used to obtain bistability. Trypsin is autocatalytically produced from trypsinogen, but deactivated by an inhibitor. **B)** Modification of the trypsin inhibitor AEBSF by guanylation of its amine. Details of the synthesis are in the Experimental Section. **C)** Inhibition of trypsin ($[Tr]_0$ = 29.4 μM) by GEBSF ($[GEBSF]_0$ = 40 μM) in 0.1 M Tris-HCl, pH 7.7, 20 mM $CaCl_2$ at 22 °C. The experimental data (dots) were fitted by a bimolecular reaction model in COPASI (dashed line). **D)** Hydrolysis of the sulfonyl fluoride of GEBSF as followed by ^{1}H NMR in 50 mM Tris-HCl, pD 7.7, in D_2O containing 20 mM $CaCl_2$ at 22 °C. The experimental data (dots) were fitted in Origin (solid line). Abbreviations: Tg = trypsinogen, Tr = trypsin, Inh = inhibitor, AEBSF = 4-(2-aminoethyl)benzenesulfonyl fluoride, GEBSF = 4-(2-guanidinoethyl)benzenesulfonyl fluoride.

outcompete autocatalysis, but at higher concentrations of trypsin, the rate of autocatalysis is increased tremendously due to its nonlinear nature, and a high concentration of trypsin is maintained.

Therefore, it is necessary for the inhibitor to strongly oppose trypsin formation (*i.e.* relatively high rate of inhibition), while allowing the trypsin concentration to quickly increase when autocatalysis outcompetes inhibition (for instance when all inhibitor has reacted). These are the properties we are looking for below, when we test two different trypsin inhibitors.

2.1. Synthesis and kinetic studies of potent inhibitor

First, we synthesized an arginine-like analogue of the commercially available trypsin inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF, compound **1** in Fig. 1b) by guanylating its amine using a standard two-step procedure (Fig. 1A).[19] In the first step, AEBSF reacts with *N,N'*-di-boc-thiourea (**2**) to form a boc-protected guanidinium group (compound **3**), after which a simple boc-deprotection step yields the desired compound 4-(2-guanidinoethyl)benzenesulfonyl fluoride (GEBSF, **4**).

Next, we investigated the inhibitory properties by mixing GEBSF (40 μM) with trypsin (29.4 μM). The activity of trypsin was measured over time by a fluorogenic assay (see the Experimental section). Fig. 1C shows that trypsin is fully inhibited after 15 min (orange dots), indicating that GEBSF is indeed a potent inhibitor of trypsin. The experimental data were fitted to a bimolecular reaction model in COPASI (Fig. 1C, orange dashed lines), and a parameter estimation procedure resulted in a rate constant $k_{inh,GEBSF}$ of 1104 mM^{-1} h^{-1}. This value is much higher than the trypsin inhibition rate constant for AEBSF (53 mM^{-1} h^{-1}) we measured before, but also considerably larger than the k_{cat}/K_M value (a measure of enzymatic efficiency) of trypsinogen conversion by trypsin (63 mM^{-1} h^{-1}).[16] Therefore, it is expected that GEBSF would be more suitable to antagonize autocatalytic trypsin production than AEBSF.

Hydrolysis of the sulfonyl fluoride moiety is a side reaction that needs to be measured as well, as it results in a sulfonic acid that inhibits trypsin only weakly and reversibly. To that end, GEBSF was dissolved in deuterated buffer, and changes in the benzene ring peaks due to hydrolysis were monitored by ^{1}H NMR at 22 °C. The reaction was considered to be pseudo-first order, and a linear fit through experimental data points expressed as ln([GEBSF]/[GEBSF]$_0$) yielded a hydrolysis rate constant $k_{hyd,GEBSF}$ of 0.044 h^{-1} ($t_{½}$ = 15.8 h, Fig. 1D). This value is comparable with the hydrolysis rate constant for AEBSF (0.034 h^{-1}),[16] and will be taken into account in all computational models mentioned below. The weak, reversible inhibition of trypsin by the hydrolyzed inhibitor is not considered in the models, as initial calculations showed no significant effect thereof on the behavior of the network.

2.2. Batch experiments and modelling

Next, we tested the properties of the inhibitors in batch experiments in which thermodynamic equilibrium is inevitably reached. Trypsinogen (100 μM) is fully converted into trypsin within two hours in the absence of inhibitor, and the S-shaped activation curve is characteristic for autocatalytic reactions (orange dots in Fig. 2). The activation of trypsinogen is severely delayed in the presence of AEBSF (50–100 μM), and the S-shaped curve is much more gradual than in the absence of inhibitor (top panel in Fig. 2). The experiment with 100 μM AEBSF still yields a final trypsin concentration of about 40 μM due to significant hydrolysis of the inhibitor at this timescale. In contrast, lower concentrations (5–9 μM) of GEBSF are required to obtain a similar delay in activation as with high concentrations of AEBSF, because of the higher inhibition rate constant of GEBSF. Consequently, higher final concentrations of trypsin are obtained when GEBSF is used (bottom panel in Fig. 2). Moreover, in case of GEBSF the steepness of the activation curves strongly resembles the one in the absence of inhibitor.

Then, we built a model describing these batch experiments in COPASI to gain more insight in the differences between the inhibitors. In the model, four reactions are considered: 1) autoactivation of trypsinogen, 2) activation of trypsinogen by trypsin, 3) inhibition of trypsin by an inhibitor, 4) hydrolysis of the inhibitor. A