SUPPLEMENT

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Understanding thermal sensitivity at the molecular level and developing temperature-based systems using RNA Thermometers

Abstract

Purpose: Temperature sensitivity is found in all multicellular organisms, as well as in most primitive life forms. The ubiquity of this temperature sensitivity is an indicator of its effects at the multicellular, cellular and molecular levels [1]. Previous studies have shown that temperature-based regulation is present in the transcriptional process [2]. RNA Thermometers, temperature-sensitive sequences, have been shown to act on heat-shock genes to regulate temperature-dependant systems in many organisms [3,4]. The goal of this study was to characterize the shifts in the functioning of these RNA Thermometers at various temperatures. In addition, using the principle of transcriptional thermoregulation, an automated temperature-responsive system stimulating inverse endothermic and exothermic enzymatic reactions for heat stabilization was proposed.

Methods: The endothermic enzymatic reaction was designated as the breakdown of urea, reflecting the function of urease, and the exothermic reaction was designated as the breakdown of hydrogen peroxide, reflecting the function of catalase [5].

Results: The proposed system was built upon the translation of urease and the inhibition of catalase translation at higher temperatures, and the inverse at lower temperatures. As RNA Thermometers can be used only to drive transcription at higher temperatures, the installation of a lac-regulated 2-way system was suggested. This system would also provide a synthetic solution to thermoregulation and the current systems employed today. This system could be applied where the current thermoregulatory systems prove insufficient and could be further developed and optimized to replace them in the future.

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Life necessitates a struggle between life forms and nature. Organisms have developed homeostasis as a method of maintaining a structural integrity independent from nature. Although a wide range of homeostatic mechanisms exist, they cannot always provide the necessary conditions; sometimes organisms must change in response to their environmental conditions – such as temperature – in order to adapt. Thermoregulatory systems have proven to be significant from both evolutionary and social standpoints. As such, the RNA Thermometer provides great insight into how various living organisms function in response to heat stimulus [6]. It also provides a basic sensory mechanism for use by life scientists.

The purpose of this study was to address the applicability of the RNA Thermometer at varying temperatures. The secondary goal of this study was to construct a biological thermoregulatory system that utilizes the function of the RNA Thermometer by linking it to the regulation of enzymatic activity at the cellular level. Two enzymes with marginal enthalpy values were designated to be used for the enzymatic function: urease was selected for its high endothermic properties [7,8] and catalase was selected for its significant exothermic potential [9]. These enzymes have relatively basic substrates, making them widely applicable as an alternative to the current industrial thermoregulatory systems.

RNA Thermometers

RNA Thermometers are a group of RNA structures that have varying properties and operative functions. Although RNA Thermometers are relatively simple structures and are found in almost all organisms, conformational, structural, functional and mechanical differences exist between RNA Thermometers found in different species. Nevertheless, there are certain features that are characteristic of RNA Thermometers. All RNA thermometers function through conformational (structural) shifts. Conformational shifts expose the Shine-Dalgarno (SD) sequence, which is present in all RNA Thermometers and which acts as a ribosomal binding site to initiate translation. Temperature is the main factor responsible for the structural change, and both cold-shock and heat-shock RNA Thermometers exist [10].

The RNA Thermometer that is the focus of this study is the *ibpB* RNA Thermometer. Found in all strains of *Escherichia coli*, the *ibpB* Thermometer is a standard cis-acting heat-shock-regulated RNA Thermometer. The RNA Thermometer stimulates activity starting at 32°C; however, optimal unbinding of the DNA sequence reaches a maximum at 37°C [11]. This temperature-dependence can be primarily attributed to a Maxwell-Boltzman energy distribution at the

molecular level. The rate of the reaction and the translation increases further as the temperature passes the optimum point, as the number of ribosomes that can attach to the SD sequence increases with temperature – with the temperature of denaturation being the limiting factor.

Assay for *ibpB* Thermometer Functionality

A recombinant DNA part was assembled to test the functionality of the thermometer. A T7 promoter was attached to the *ibpB* Thermometer as the RBS sequence and a RFP downstream as the reporter. The vector was cut using EcoRI and HindIII restriction enzymes and verified. Sample 7-6 on the cloned vector can be seen holding the necessary base pair length.

Ten samples of 5 ml of transformed *E. coli* cultures were then incubated at 37°C for 13 hours. To five of the ten samples was then added 50 uM of IPTG to induce the T7 promoter with a period of 4 hours twice and further incubated at respective temperatures (with the other samples acting as control groups) to test functionality at different temperatures. The RFP in the cultures were then isolated and compared statistically.

Alternative Thermoregulatory System

The proposed system is composed of two functional systems acting against one another: the urease system, which is the primary system, is active at higher temperatures and the catalase system is active at lower temperatures. The two systems are meant to function inversely, as they have opposite functional roles. The enzymes were selected taking into account their enthalpy properties and relatively simple substrate properties. Urease has a high Gibbs Free Energy value [7,8] and catalase has a significantly high energy release per mol of reaction [9]. Urease is the ideal cooling enzyme and catalase is the ideal heating enzyme.

The regulation of the two systems has to shift with temperature changes to provide a 'smart' thermoregulator system. Through the installation of an RNA Thermometer into the sequence of the systems as an RBS, the protein translation of urease and catalase can be managed with temperature. Of the two systems, the urease system is to be regulated directly by the RNA Thermometer, as urease is the cooling enzyme expressed at elevated temperatures.

The catalase system will require increased production levels as the temperature decreases, hence a heat-shock RNA Thermometer will not function for the catalase system. With the installation of a lac operon to inhibit catalase production,

FIGURE 1. The electrophoresis result for the vector listed in Figure 1, the vector was cut with EcoRI and HindIII enzymes and two segments were formed with one 1100 bp long and the other too miniscule to observe. The result is labeled as 7,6

FIGURE 2. The graph shows the variance in density concentrations of RFP, caused as a result of the functioning of the IPTG Inducible Promoters in different temperatures. The temperatures in which activity were tested are 4°C, 25°C, 37°C, 41°C and 50°C. Data on RFP concentration were acquired at 584 nm of emission and 607 nm of excitation spectra fluorescence spectroscopy. The vertical axis is for protein concentration and the horizontal axis is for respective temperature. As it can be seen, the functioning of the RNA Thermometer increased with increased temperatures.

the need for an RNA Thermometer on both systems is eliminated. The lac operon will allow the inverse functioning of the two systems. The lacI attached after the RNA Thermometer will inhibit the catalase system by inhibiting the lacO. This will close the heating process at higher temperatures. At lower temperatures, the RNA Thermometer will shift to a closed structure, also stopping lacI stimulation and allowing the lacO-affected catalase system to operate. With this construct, the complete system will have two main functional stimulationates at the two temperature levels. Temperatures below 32 °C will show no RNA Thermometer function as an RBS and the urease and lacI systems will be closed to translation. The catalase will be freely produced allowing for efficient heating. In temperatures above 32 °C, the RNA Thermometers will be open and the SD Sequence exposed allowing for urease production alongside lacI. Catalase production will be obstructed allowing for efficient cooling.

FIGURE 3. Representation of the mechanism below 32°C, lacI and urease will not be produced in the absence of RNA Thermometer activity and catalase will be freely produced in the absence of lacI.

FIGURE 4. Representation of the system above 32°C, the function of the RNA Thermometer will stimulate lacI and urease expression, which, in turn, will have catalase production under inhibition by lacI.

Discussion

The assays done on the RNA Thermometer confirmed that the *ibpB* RNA Thermometer functioned at the temperature values described in the literature [12]. The characterization experiments showed that temperature values below 32°C had no significant RFP production, suggesting that the RNA Thermometer was structurally dysfunctional. The experiments also showed that there was an increase in function as the temperature increased from 32°C. The increased function of the thermometer at temperatures above 37°C, which is supposedly the conformationally optimum temperature, can be explained with increased kinetic activity at the molecular level: as long as the proteins are not yet denatured, they will exhibit increased reaction kinetics with increased temperature. The cultured *E. coli* strains remain functional up to 51°C, meaning that the enzyme activity should continue to increase. The cloning for the proposed thermoregulatory system was successfully conducted but results were not included here due to the lack of full characterization. This system, however, holds promise as an alternative to those currently in use; providing biologically-relevant data in environments supportive of living organisms.

Interest in regulatory mechanisms continue to foster science research. Temperature regulation at the molecular level is an asset that could be utilized in many areas of life science, just as pH and chemical induction are. Furthermore, thermoregulatory systems built on biological constructs could be further improved and developed to, perhaps one day, replace the very machines that commonly reside in our homes today.

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