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Identifying the conditions necessary for the thioredoxin ultrasensitive response[☆]

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Summary   Thioredoxin, glutaredoxin, and peroxiredoxin systems (collectively called redoxins) play critical roles in a large number of redox-sensitive cellular processes. These systems are linked to each other by coupled redox cycles and by common reaction intermediates into a larger network.

Previous results from a realistic computational model of the *Escherichia coli* thioredoxin system developed in our group have revealed several modes of kinetic regulation in the system. Amongst others, the coupling of the thioredoxin and peroxiredoxin redox cycles was shown to exhibit the potential for ultrasensitive changes in the thioredoxin concentration and the flux through other thioredoxin-dependent processes in response to changes in the thioredoxin reductase level. Here, we analyse the basis for this ultrasensitive response using kinetic modelling and metabolic control analysis and derive quantitative conditions that must be fulfilled for ultrasensitivity to occur.

Introduction

Thioredoxins, glutaredoxins and peroxiredoxins (collectively termed redoxins) are present in all living organisms and regulate a diverse array of redox-sensitive processes including DNA synthesis, transcription factor activation, anti-oxidant defence (Toledano et al., 2007) and signal transduction (Finkel, 2011; Winterbourn and Hampton, 2008). In these reactions, redoxins are oxidized by their targets and are therefore coupled to additional redox cycles forming the thioredoxin, glutaredoxin and peroxiredoxin systems, which are linked into an integrated network (Pillay et al., 2013).

This network is complex both in terms of its size and interconnectivity and dysregulation of the network is associated with a number of diseases including cancer, HIV susceptibility, heart disease and several neurodegenerative disorders (Holmgren and Lu, 2010). Further, pathogen redoxin systems are essential for survival in infectious diseases such as tuberculosis and malaria and these systems are consequently being evaluated as drug targets (Jaeger and Flohé, 2006). Computational systems biology approaches could play an important role in precisely delineating roles played by redoxin networks in these pathologies and in identifying druggable targets (Pillay et al., 2013). However, progress here has been limited by the conflicting descriptions of redoxins in the literature and inconsistencies in the quantitative measures and kinetic models of redoxin activity (Pillay et al., 2009, 2013).

Our recent work has resolved several of these contradictions. Using kinetic modelling, we showed that the enzyme-like behaviours (e.g. substrate saturation) attributed to thioredoxins resulted from redistribution of oxidised and reduced thioredoxin in the thioredoxin cycle, and are not due to enzymatic properties of thioredoxin itself (Pillay et al., 2009). Moreover, we described how redoxin system dynamics can result in regulatory designs that cannot be anticipated by studying system components in isolation (Mashamaite et al., 2015).

The insights from our work resulted in the first *in vivo* models of redoxin networks by us and other groups (Adimora et al., 2010; Benfeitas et al., 2014; Pillay et al., 2011). We developed the first realistic model of the *Escherichia coli* thioredoxin system (see Fig. 1). In contrast to the view that the thioredoxin network was analogous to an electrical circuit, we showed this system was interconnected, displayed adaptability and described a novel form of ultrasensitivity (Pillay et al., 2011).

The main result from our previous study was that the reduced thioredoxin concentration (TrxSH), and as a consequence the flux through certain TrxSH-dependent reactions, could respond in an ultrasensitive manner to changes in the levels of thioredoxin reductase (Fig. 2). Here, ultrasensitivity is defined as a slope of greater than one when the response is plotted against the input in double-logarithmic space.

Ultrasensitivity is normally associated with signalling and together with other findings (Finkel, 2011), it is emerging that reactive oxygen species, particularly hydrogen peroxide, are important cellular secondary messengers in signalling, even under normoxic conditions (Winterbourn and Hampton, 2008). However, the quantitative signal
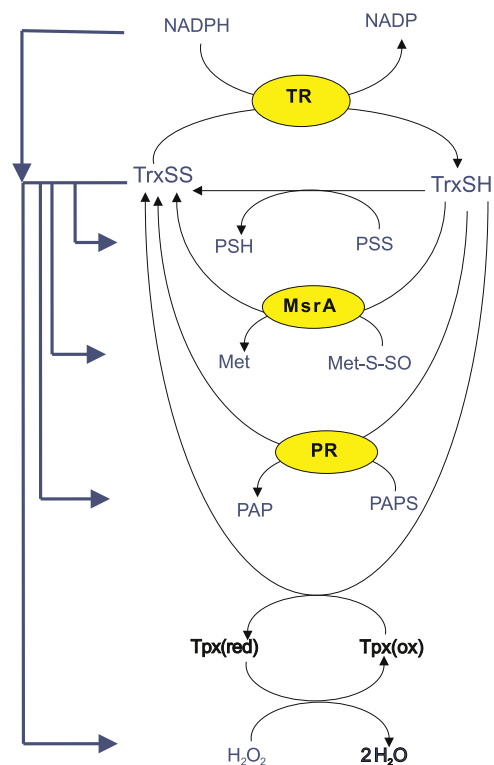

Figure 1 Reaction scheme of the kinetic model of the *E. coli* thioredoxin system. Blue arrows denote electron flow. Abbreviations: TrxSS and TrxSH, oxidised and reduced thioredoxin respectively; TR, thioredoxin reductase; PSS, oxidised protein, Met-S-SO, methionine sulphoxide; MsrA, methionine sulphoxide reductase; PAPS, phosphoadenosine phosphosulphate; PR, PAPS reductase; Tpx, peroxiredoxin. Taken from Pillay et al. (2011).

properties, targets and dynamics of this process are not clear and our understanding of the balance between redox signalling and redox stress is vastly incomplete. In this paper, we use kinetic modelling and metabolic control analysis to elucidate the mechanistic basis for the ultrasensitive response shown in Fig. 2.

Methods

Metabolic control analysis

Metabolic control analysis (MCA) is a framework for quantifying the control properties of a steady-state metabolic system in terms of the responses of the system fluxes and metabolite concentrations to perturbations in the rates of the reactions (Kacser et al., 1995; Heinrich and Rapoport, 1974). For this purpose, MCA defines two types of coefficients:

- An *elasticity coefficient* describes the sensitivity of an individual reaction rate towards a change in any concentration x of a substrate, product, or effector that affects the reaction directly. It is defined as the ratio of
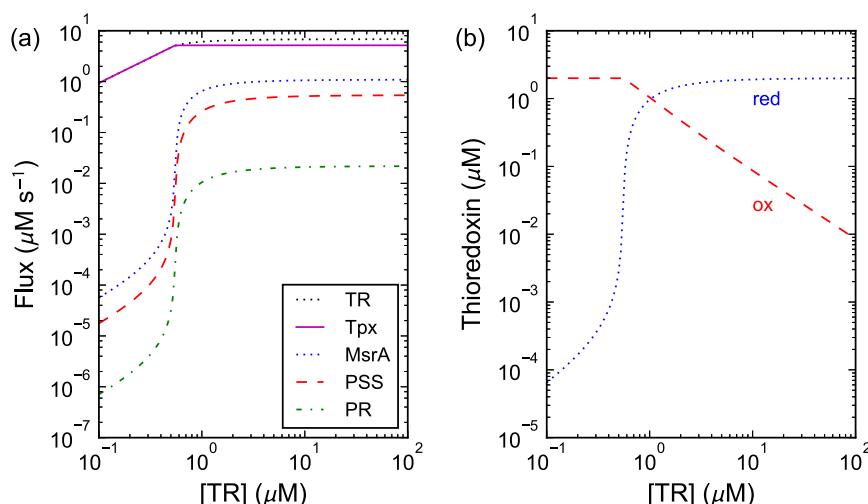
Figure 2 Effect of changes in the thioredoxin reductase levels on fluxes through the TrxSH-dependent demand reactions (a) and the concentrations of reduced and oxidised thioredoxin (b). The total concentration of thioredoxin reductase was varied over three orders of magnitude in a computational model of the *E. coli* Trx system, and the fluxes and concentrations determined from the model output. Lines are labelled according to the abbreviations in Fig. 1. Adapted from Pillay et al. (2011).

the relative change in the rate of reaction *i* (denoted v_i) to the relative change in *x*:

$$\varepsilon_x^{v_i} = \frac{\partial \ln v_i}{\partial \ln x} \tag{1}$$

- A *control coefficient* describes the sensitivity of a steady-state system variable (flux or concentration), towards a change in a local reaction rate. The control coefficient is defined as the ratio of the relative change in a system variable *y* to the relative change in a reaction rate, v_i:

$$C_{v_i}^y = \frac{d \ln y}{d \ln v_i} \tag{2}$$

where *y* can be a steady-state flux, concentration, or a function thereof (e.g. a concentration ratio). The use of a total derivative in this case signifies that the entire system is allowed to relax to a new steady state after the perturbation in v_i, and control coefficients are thus system properties.

One of the key results of MCA, which will be used in this work, is that it allows control coefficients (systemic properties) to be expressed in terms of elasticities (local properties) (see e.g. Heinrich and Schuster, 1996; Hofmeyr, 2001).

Simplification of the network

To simplify the mathematical and computational analysis, the model in Fig. 1 was reduced by removing all thioredoxin demand reactions except for the peroxiredoxin system and a single additional demand reaction, i.e. PSS reduction (Fig. 3(a)). Redoxin systems are networks of interlinked moiety-conserved cycles (Fig. 3(a)), and the MCA of such systems quickly yields complex expressions containing a mix of concentrations and elasticities (Hofmeyr et al., 1986). However, this can be simplified dramatically by considering concentration ratios as variables instead of
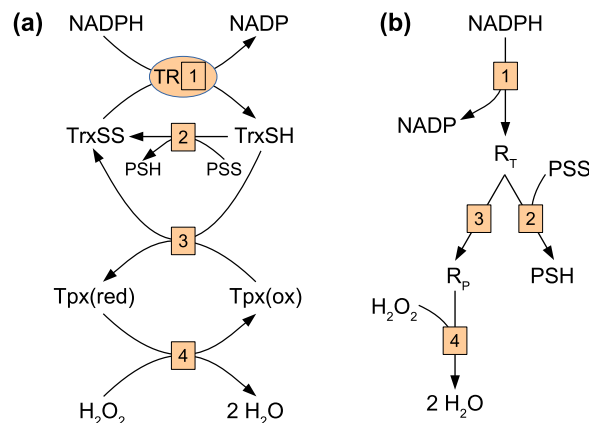

Figure 3 Reaction scheme of the reduced thioredoxin model. (a) Moiety couples are drawn explicitly. (b) The model in terms of redoxin ratios, yielding a branched pathway with linear sections. Abbreviations are as in Fig. 1, R_T and R_P are defined in the main text. Reaction numbers are indicated in square boxes.

the concentrations themselves (Westerhoff and van Dam, 1987; Hofmeyr and Rohwer, 1998), which in effect leads to a linearisation of the system (Fig. 3(b)).

In this specific case, we define $R_T = TrxSH/TrxSS$ (the thioredoxin ratio) and $R_P = Tpx_{red}/Tpx_{ox}$ (the peroxiredoxin ratio). Denoting the total thioredoxin concentration by S_T and the total peroxiredoxin by S_P, the concentrations of the individual members of the moiety couple can be calculated as follows:

$$TrxSS = \frac{S_T}{R_T + 1} \tag{3}$$

$$TrxSH = \frac{R_T S_T}{R_T + 1} \tag{4}$$

The expressions for Tpx are similar.

Computational analyses

Numerical simulations of the model, as well as computation of elasticities and control coefficients, were performed with the PySCeS (Python Simulator for Cellular Systems) software (Olivier et al., 2005). Symbolic control analysis was performed with the symca tool from the PyscesToolbox (https://github.com/PySCeS/PyscesToolbox, Christensen et al., 2015). Computations were performed in the IPython notebook environment (http://ipython.org, Pérez and Granger, 2007).

Results and discussion

Ultrasensitivity in a simplified model

In order to simplify the analysis and to elucidate the mechanistic origin of the observed ultrasensitivity, we analysed a simplified model of the *E. coli* thioredoxin network that only contained two thioredoxin demand reactions, viz. peroxiredoxin and protein disulphide reduction (Fig. 3). In previous results ((Pillay et al., 2011), see also Fig. 2) ultrasensitivity was observed in all thioredoxin-dependent reactions except for Tpx reduction. When considering only one of these (PSS reduction), ultrasensitivity was still observed (Fig. 4(a)), validating the use of the simplified model for further exploration.

Our previous work had also shown that the presence of ultrasensitivity depended on a particular constellation of rate constants for Tpx reduction (k_3) and Tpx oxidation (k_4). Specifically, when the ratio k_3/k_4 was decreased, the degree of ultrasensitivity decreased until it was abolished completely (Pillay et al., 2011). This effect could be reproduced with the simplified model (Fig. 4(b) and (c)), where a decrease in k_3 (and as a consequence a decrease in k_3/k_4) led to an attenuation in the ultrasensitive response of J_2 towards changes in thioredoxin reductase until it vanished.

A similar effect was observed when k_3/k_4 was decreased by increasing k_4 (data not shown).

Branch-point ultrasensitivity

The concept of 'branch-point ultrasensitivity' was first introduced by Koshland and co-workers (LaPorte et al., 1984; Walsh and Koshland, 1985). They studied branched metabolic systems in which the flux through one of the branches responded in an amplified fashion to changes in the input flux. In order to quantify this effect, Koshland et al. (1982) defined a 'sensitivity amplification factor', which compares the relative (or percentage) change in a response to the relative change in stimulus which caused that response. This definition is similar to that of a flux-control coefficient (Eq. (2)), with the stimulus referring to changes in the local reaction rate v_i and the response to the flux as steady-state variable y.

LaPorte et al. (1984) identified as one of the necessary conditions for amplification the requirement that the K_M values of the committing enzymes in each branch for the branch metabolite differ. Ultrasensitivity can then be observed in the branch with the higher K_M value under some conditions where the enzyme will be sensitive towards the metabolite while the other enzyme is insensitive (saturated). Koshland's branch-point analysis was cast in the framework of MCA (Rohwer, unpublished work). In terms of MCA, the difference in K_M values translates to a difference between the elasticities (Eq. (1)) of the two branch-point enzymes for the branch metabolite.

In terms of the system in Fig. 3(b), the ultrasensitive response shown in Fig. 4 can therefore be quantified in terms of the control coefficient $C_1^{J_2}$. Fig. 5 shows the values of this control coefficient with varying thioredoxin levels for the three values of k_3. In the reference model, a control coefficient value of almost 50 was observed in the area of the steepest flux response, which resulted in almost switch-like behaviour.
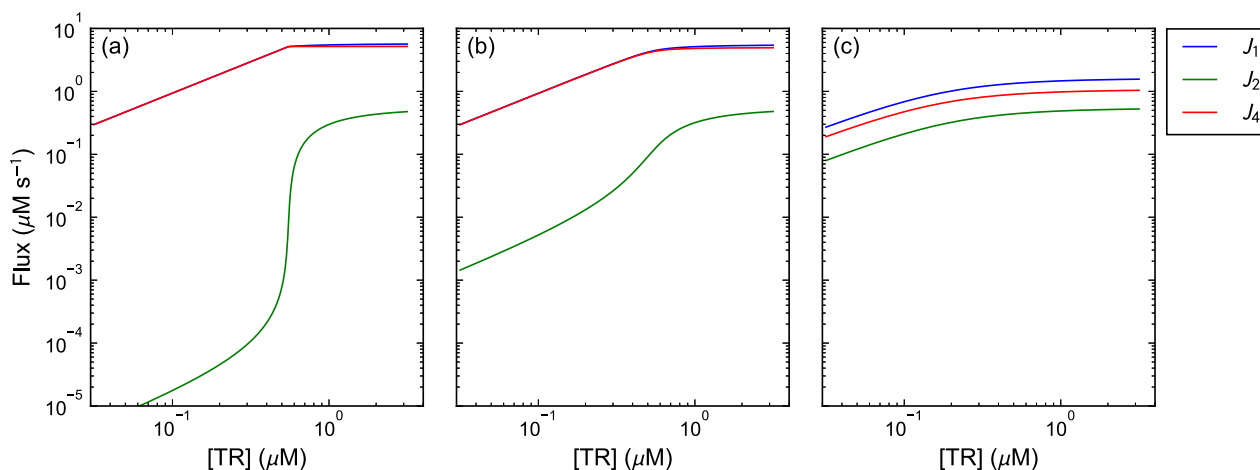
Figure 4 Effect of changes in the thioredoxin reductase concentration on the fluxes in the simplified model. Reactions are numbered as in Fig. 3. The fluxes through thioredoxin reductase (J_1), PSS reduction (J_2) and the Tpx system (J_4) are shown. Note that $J_3 = J_4$ in steady state, so that only J_4 is shown. The value of k_3 was changed as follows: (a) 3000 $\mu M^{-1}\,s^{-1}$, (b) 10 $\mu M^{-1}\,s^{-1}$, (c) 0.114 $\mu M^{-1}\,s^{-1}$.
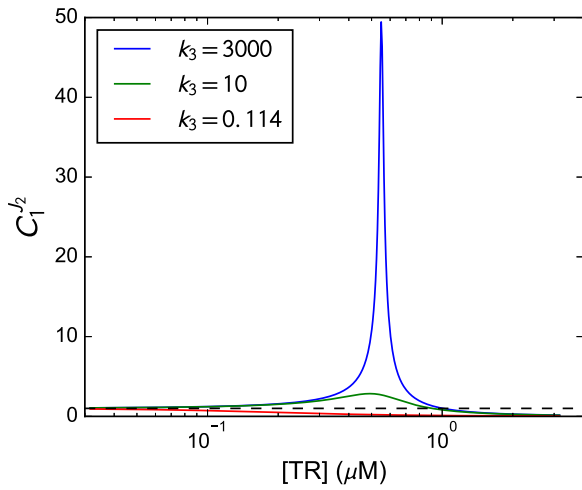
Figure 5 The branch-point control coefficient as a function of varying thioredoxin reductase levels. The value of k_3 in the model is indicated on the figure legend. A horizontal dashed line indicates a reference value of one, above which amplification is observed.

Interestingly, the second moiety-conserved cycle of peroxiredoxin was required (in addition to the thioredoxin cycle) to observe ultrasensitivity. Since all the thioredoxin demand reactions were modelled with mass-action kinetics, it was impossible for two different branches to exhibit different elasticities for R_T (the elasticities will always be the same) and hence different sensitivities to the branch metabolite (see above). We constructed such a model and indeed did not observe ultrasensitivity (data not shown).

Deriving conditions for ultrasensitivity

From the definition of control coefficients, an ultrasensitive response in J_2 will be observed if $C_1^{J_2} > 1$, i.e. the slope of the J_2-curve in Fig. 4 is >1. We used the symca package (Rohwer et al., 2008) of the PyscesToolbox (Christensen et al., 2015) to generate a symbolic expression for this control coefficient in terms of elasticities:

$$C_1^{J_2} = \frac{-J_1 \varepsilon_{R_T}^{v_2} \varepsilon_{R_P}^{v_3} + J_1 \varepsilon_{R_T}^{v_2} \varepsilon_{R_P}^{v_4}}{J_1 \varepsilon_{R_T}^{v_1} \varepsilon_{R_P}^{v_3} - J_1 \varepsilon_{R_T}^{v_1} \varepsilon_{R_P}^{v_4} - J_2 \varepsilon_{R_T}^{v_2} \varepsilon_{R_P}^{v_3} + J_2 \varepsilon_{R_T}^{v_2} \varepsilon_{R_P}^{v_4} + J_4 \varepsilon_{R_T}^{v_3} \varepsilon_{R_P}^{v_4}}$$

(5)

Using the steady-state flux relationship $J_1 = J_2 + J_4$, a sufficient condition for observing ultrasensitivity can be derived by substituting the RHS of Eq. (5) into $C_1^{J_2} > 1$:

$$-\frac{J_1 \varepsilon_{R_T}^{v_1}}{J_4 \varepsilon_{R_T}^{v_2}} + \frac{\varepsilon_{R_T}^{v_3} \varepsilon_{R_P}^{v_4}}{\varepsilon_{R_T}^{v_2} \left(\varepsilon_{R_P}^{v_4} - \varepsilon_{R_P}^{v_3} \right)} < 1$$

(6)

From this, two necessary conditions can be derived that must both be fulfilled simultaneously in order for ultrasensitivity to occur:

$$\text{Condition 1}: \quad -\frac{J_1 \varepsilon_{R_T}^{v_1}}{J_4 \varepsilon_{R_T}^{v_2}} < 1$$

(7)

$$\text{Condition 2}: \quad \frac{\varepsilon_{R_T}^{v_3} \varepsilon_{R_P}^{v_4}}{\varepsilon_{R_T}^{v_2} \left(\varepsilon_{R_P}^{v_4} - \varepsilon_{R_P}^{v_3} \right)} < 1$$

(8)

Fig. 6 analyses the amplification condition in greater detail. The figure clearly indicates that amplification only occurs if the sum of the two terms in Eq. (6) is <1 (Fig. 6(a–c)). At a high rate constant for peroxiredoxin reduction ($k_3 = 3000 \, \mu M^{-1} \, s^{-1}$, leading to strong ultrasensitivity), the value of $C_1^{J_2}$ is initially determined by Condition 2 at low thioredoxin reductase levels, but as thioredoxin reductase increases, after the maximum value of $C_1^{J_2}$ is observed, Condition 1 starts to dominate. The curves for the two conditions do not overlap; one of them is zero when the other one is non-zero, and the intersection of the lines (close to a y-axis value of zero) coincides with the maximal value of $C_1^{J_2}$.

As the k_3 value is decreased, two effects become apparent. First, the line representing Condition 1 shifts to the left to cross the threshold of 1 at a lower thioredoxin reductase concentration, and second, the line representing Condition 2 decreases to a lesser extent with increasing thioredoxin reductase. As a result, the sum of the two conditions is greater overall and moreover <1 over a narrower thioredoxin reductase-range, leading to a drastically reduced $C_1^{J_2}$-value. When $k_3 = 0.114 \, \mu M^{-1} \, s^{-1}$, the sum of the two conditions is >1 over the complete range of thioredoxin reductase concentrations, even though both conditions are individually <1 at low thioredoxin reductase values. While both necessary conditions are satisfied, the sufficient condition (Eq. (6)) is not, and hence no amplification is observed.

Each of the two necessary amplification conditions was explored in greater detail by considering the factors making up the expression. Condition 1 is virtually completely dominated by $-\varepsilon_{R_T}^{v_1}/\varepsilon_{R_T}^{v_2}$, as $J_1/J_4 \approx 1$ over the whole range of thioredoxin reductase-values (Fig. 6(d–f)). Because the Tpx flux (J_4) is much greater than the PSS reduction flux (J_2), any increase in the latter as a result of increasing thioredoxin reductase levels would have hardly any effect on the total flux (J_1). Fig. 6(d–f) therefore shows that for amplification to occur, $-\varepsilon_{R_T}^{v_1}/\varepsilon_{R_T}^{v_2}$ must be <1, i.e. $|\varepsilon_{R_T}^{v_1}| < \varepsilon_{R_T}^{v_2}$. In biological terms this means that the PSS reduction reaction must be *more sensitive* to changes in the thioredoxin ratio than the thioredoxin reductase reaction.

Similarly, Condition 2 was split into its constituent factors (Fig. 6(g–i)). A number of interesting observations emerge. First, Condition 2 is completely determined by $\varepsilon_{R_P}^{v_4}$. The other factors in the term cancel out because $\varepsilon_{R_T}^{v_3}/\varepsilon_{R_T}^{v_2} = 1$ and $\varepsilon_{R_P}^{v_4} - \varepsilon_{R_P}^{v_3} = 1$ (the red solid line and black dashed line coincide). Second, Condition 2 ($\varepsilon_{R_P}^{v_4}$) varies between 1 and 0, and as a result can never prevent ultrasensitivity from occurring *on its own*. However, since it is the sum of Conditions 1 and 2 that ultimately determines if ultrasensitivity will occur (Eq. (6)), $\varepsilon_{R_P}^{v_4}$ can contribute by pushing this sum over a value of one (Fig. 6(c)) at low thioredoxin reductase values. In biological terms $\varepsilon_{R_P}^{v_4}$ refers to the sensitivity of the hydrogen peroxide reduction reaction to changes in the peroxiredoxin ratio. Therefore, the more sensitive this reaction, the less likely is the chance for ultrasensitivity to be observed. In this regard it is interesting to note that the intracellular total peroxiredoxin concentration is very high, and Pannala and Dash (2015) recently showed that high peroxiredoxin concentrations were needed to observe ultrasensitivity in a kinetic model. The authors modelled peroxiredoxin with a detailed enzymatic mechanism (Pannala and Dash, 2015). In contrast, we (Pillay et al., 2011) and others (Adimora
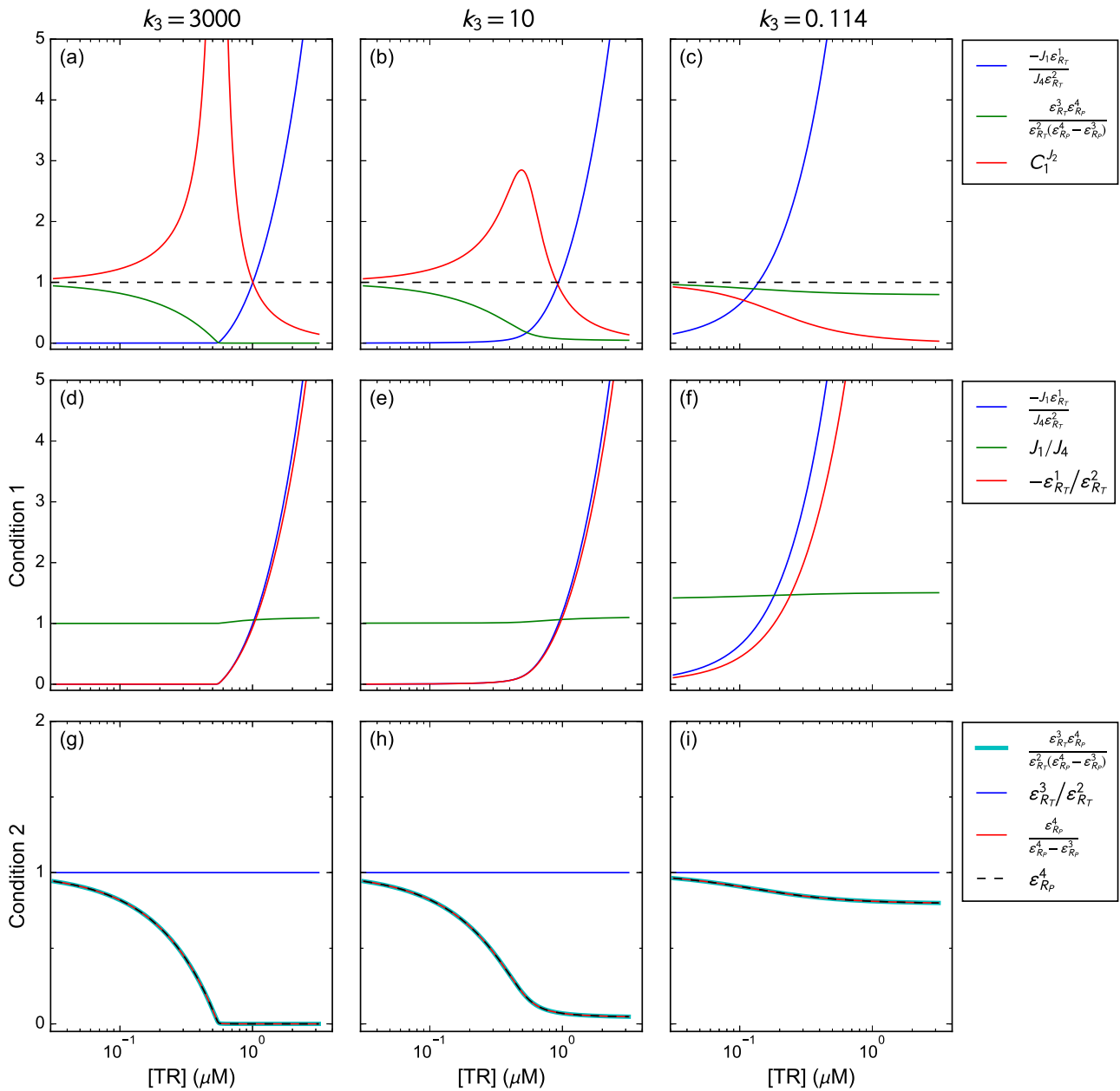
Figure 6   Exploring the terms of the amplification condition (Eq. (6)) with varying thioredoxin reductase levels. The values for k_3 (in $\mu M^{-1}\,s^{-1}$) in the model were adjusted as indicated at the top of each column to yield conditions leading to strong, moderate and no ultrasensitivity. Control coefficient and elasticity values were calculated with PySCeS. (a–c) The amplification control coefficient as well as the two terms of the amplification condition. Condition 1 (Eq. (7)) is plotted in blue and Condition 2 (Eq. (8)) in green. (d–f) Condition 1 (Eq. (7)) and its constituent factors. (g–i) Condition 2 (Eq. (8)) and its constituent factors.

et al., 2010) have used mass action kinetics to describe per-oxiredoxin because the resulting models described the data sufficiently, the second-order rate constants could be readily measured *in vitro*, and the peroxiredoxin concentrations are much greater than the hydrogen peroxide concentrations *in vivo*. Since we used our earlier model (Pillay et al., 2011) as a basis for the current analysis and wanted to compare results, we continued to model peroxiredoxin kinetics with mass action in this paper.

Conclusion

In this paper we have elucidated the mechanistic basis for the ultrasensitive flux-response observed in a kinetic model of the *E. coli* thioredoxin system in response to varying thioredoxin reductase levels. Using MCA, we have derived conditions that must be fulfilled before amplification can occur. The most important conclusions are:

- A second linked moiety-conserved cycle (such as the peroxiredoxin system) in addition to the primary thioredoxin cycle is required to observe ultrasensitivity.
- For any thioredoxin demand reaction flux to respond in an ultrasensitive manner, this reaction needs to be more sensitive to changes in the thioredoxin ratio than thioredoxin reductase.
- The less sensitive the hydrogen peroxide reduction reaction is to changes in the peroxiredoxin ratio, the greater the chance of observing ultrasensitivity.

It has become clear that oxidants such as hydrogen peroxide play a key role as secondary messengers in insulin regulation, but also enhance phosphokinase signalling and may themselves be signal molecules. However, despite its importance, redox signalling is still a loosely defined concept in the literature (reviewed in Finkel (2011)) and it is not clear what the redox signal is or how it can be measured. The ultrasensitive response analysed in this paper is a characteristic signature of signalling. Current work in our group centres on developing a framework for quantitatively describing redoxin signalling, which would allow us to distinguish quantitatively between redox stresses and redox signals that can result distinct cellular fates and to predict these signalling events with the aid of kinetic models.

Acknowledgements

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