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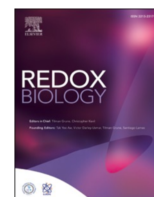

Flux versus poise: Measuring the dynamic cellular activity of the thioredoxin system with a redox probe

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Many biochemistry textbooks tend to emphasize the energy state of the cell as related by the ATP/AMP ratio and how this important marker of the energy status of the cell governs ATP consuming and ATP producing metabolic processes (through regulation of the activity of AMP kinase for example). Less attention is paid to how the redox state of the cell regulates the activity of many metabolic processes as reflected by the NADH/NAD^+ ratio and the GSH/GSSG ratio. Even less attention is paid to how these latter two ratios are altered by various metabolic states.

The redox state is greatly altered in many diseases such as cancer, diabetes, and viral infection. For example, mitochondrial dysfunction in many cancer types leads to enhanced production of superoxide, which rapidly dismutates into H_2O_2. This increase in H_2O_2 production signals the cell to change from a relatively quiescent state to rapid cell division [1]. This signaling happens because many of the kinases and phosphatases that students are taught about in their general biochemistry course are regulated by the redox status of their cysteine residues [2]. Determination of the redox state of the cell as reflected by the GSH/GSSG ratio, or the ratio of reduced to oxidized cysteine residues in a redox active protein reflects the *redox poise* of the cell.

The GSH/GSSG ratio can be measured by various techniques such as HPLC with electrochemical detection [3], while the ratio of oxidized cysteine to reduced cysteine can be determined by a "redox Western blot" [4] or newer fluorescent protein sensors. The latter are used by constructing a fusion protein consisting of a redox active protein (thioredoxin (Trx) or glutaredoxin (Grx) for example) with a fluorescing protein whose signal depends on the redox state of its cysteine residues [5]. Since the fluorescent sensor protein is fused to one of the proteins of the major antioxidant systems, the fluorescent signal is directly proportional to the redox state of Trx or Grx.

While measuring the redox poise of the cell is informative, it only captures a static picture of the cell in a particular metabolic state. These measurements allow for no conclusions about how rapidly oxidized and reduced states are interchanging. This is the redox flux. In a recent issue of *Chem*, Thorn-Seshold and coworkers have developed a highly innovative method for measuring the flux of the thioredoxin system by developing a new fluorescent substrate for its beating heart, the selenoenzyme thioredoxin reductase (TrxR) [6]. Because the expression of TrxR is upregulated in many disease states such as cancer and diabetes [7,8], this represents a new diagnostic tool with which *altered redox flux* of the cell can be measured. The primary function of TrxR is to reduce Trx, which in turn reduces downstream targets such as peroxiredoxin, transcription factors such as p53, and methionine sulfoxide reductase among many, many others. The redox poise of these downstream targets could be individually measured, but directly measuring the activity of TrxR in a live cell will allow researchers for the first time to correlate activity of this very important antioxidant enzyme with the metabolic state of the cell.

The challenge for Thorn-Seshold and coworkers was to develop a substrate for TrxR that when reduced, induces a cleavage reaction that releases a fluorophore that is only active when liberated from the probe. This substrate had to be stable to background thiols such as GSH (at an intracellular concentration of ~5 mM), and protein dithiols such as Grx and Trx (intracellular concentration of 20 μM). In considering the design of the substrate, the authors considered that linear disulfides would be unacceptable because they would be prone to reduction by the monothiol background. Cyclic disulfides resist reduction by the monothiol background, but size of the ring is important. Five membered disulfides such as 1,2 dithialanes are strained and can be opened by the monothiol background and resist reclosure due to ring strain [9]. Six-membered ring disulfides (1,2 dithianes) are not strained and resist reduction by monothiols, but are not substrates for TrxR [10].

The solution that Thorn-Seshold and coworkers ultimately landed upon made use of the fact that TrxR prefers to reduce *electrophilic* substrates such as strained disulfides, dehydroascorbate, and selenocystine. The latter of which has been used as an assay for TrxR in cell lysates [11]. Thorn-Seshold synthesized a cyclic 6-membered selenenylsulfide probe, named "RX1", that when fully reduced, undergoes 5-*exo*-trig

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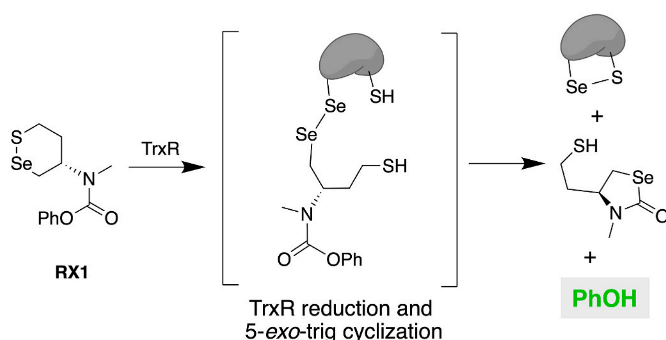

Fig. 1. Reduction of RX1 catalyzed by TrxR triggers release of a masked phenolic fluorophore. The intermediate results in a mixed deselnenide between TrxR and substrate.

cyclization (see Baldwin's rule for cyclization), forming a cyclic 5-membered selenocarbamate and releasing the masked phenolic fluorophore as shown in Fig. 1 below.

Key to the design of RX1 is inclusion of selenium in the six-membered ring. This makes the substrate electrophilic, which is the preferred site of attack by enzymic nucleophiles. TrxR itself contains a highly nucleophilic selenolate that attacks the disulfide bond of Trx and the small molecule substrates mentioned above. Attack of the enzymic selenolate of TrxR on the selenium atom of the selenenylsulfide of RX1 results in the intermediate shown above. This places the newly liberated thiol of RX1 in a less favorable position to attack the carbonyl of the carbamate (six-membered ring transition state) compared to when the selenolate is liberated upon reduction by TrxR (5-membered ring transition state). Resolution of the mixed diselenide intermediate is much faster than attack of the thiol onto the carbonyl, allowing the released selenolate to undergo 5-*exo*-trig cyclization with concomitant release of the phenolic fluorophore. The inclusion of selenium in RX1 not only makes the selenenylsulfide electrophilic and prone to attack by TrxR, but it confers resistance to reduction by monothiols because the reduction potential of the selenenylsulfide is too low (negative) to be reduced by monothiols such as GSH. This is somewhat of a paradoxical principle. Inclusion of selenium in the substrate makes the 6-membered selenenylsulfide electrophilic and prone to attack by TrxR (nearly exclusively), while conferring resistance to reduction by monothiols.

Another very interesting feature of probe design principle is revealed when Thorn-Seshold synthesized the regioisomer of RX1 in which the sulfur atom and selenium atom switched positions. This regioisomer (G) is much more prone to reduction by protein monothiols and GSH due to favorable attack at the selenium atom, liberating a thiol that can undergo 5-*exo*-trig cyclization, resulting in release of the fluorophore. The reason that RX1 is more resistant to the monothiol background is that monothiol attack at selenium results in a liberated thiol that must undergo a 6-*exo*-trig cyclization, which is much more unfavorable.

Fig. 2 illustrates the features of this truly novel redox probe. It is worth reiterating that this probe will allow for researchers to examine the redox flux of various metabolic and diseases states, which is likely to be more informative than the redox poise. It should therefore be possible to be able to build a database of the redox flux of various metabolic and disease states and use this information to develop redox based therapies. Such a database should be invaluable in developing therapies in both poorly understood diseases and newly emerging diseases.

Since probes that measure poise of the thioredoxin system, like TrxRFP1 [13], can also be used in live cells, an interesting future experiment would be to combine the use of such probes with RX1, which measures the flux. Should these two probes be used side-by-side in the

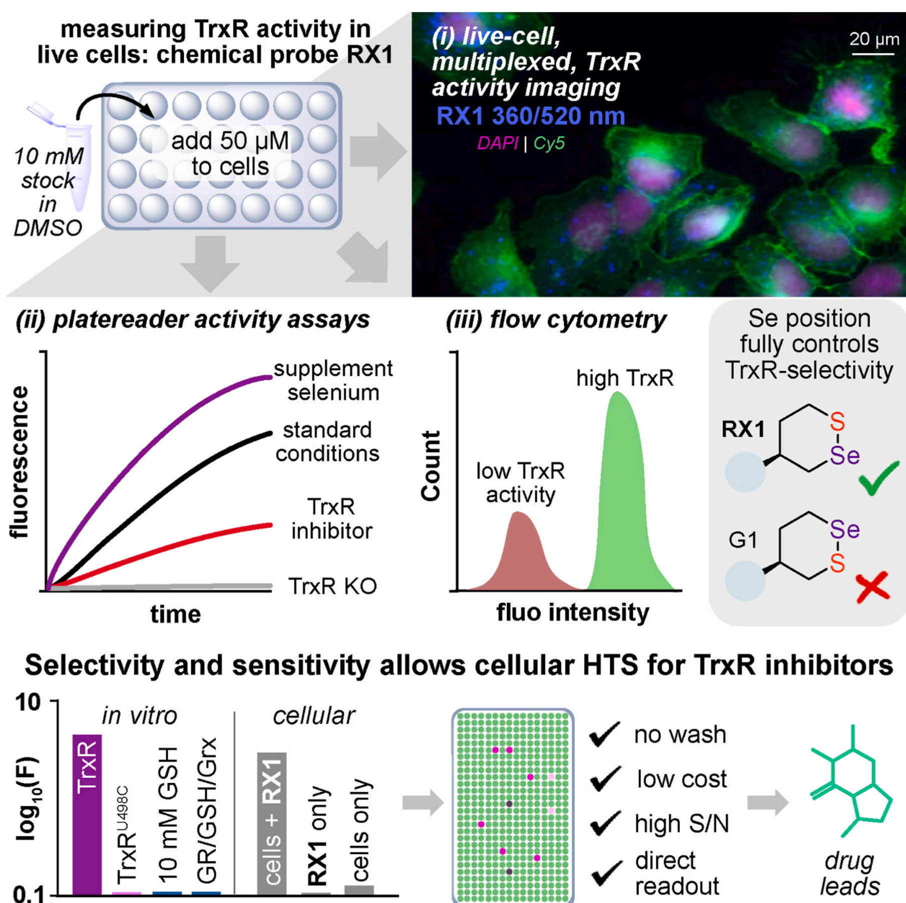

Fig. 2. The advantages of the use of RX1 to measure redox flux. The RX1 probe can be added at a reasonably low concentration (~50 μM) and used in commercial plate readers. (i) There is no need to wash or add additional reagents, just image cells live (no fixation), so can acquire live time courses. Because of its large Stokes shift it can easily be multiplexed for multi-color live imaging. Because it precipitates inside the activating cells, it can be imaged over long times without diffusing away (loss of intensity or spatial information). (ii) Its performance is what you'd expect for a quantitative and TrxR-selective probe: strongly sensitive to selenium supplementation or starvation; reports on chemical TrxR inhibitors; no signal if TrxR is knocked out, that recovers with knock-back-in. Because it precipitates, it is protected from the environment and is therefore very photostable, so long-time courses can be measured without any photobleaching. Population-average results are easy to monitor in multi-well plates (from 24- to 1536-well reported in the paper). (iii) Because it precipitates in activating cells and doesn't diffuse away, cells can also be analyzed by flow cytometry for collecting single-cell statistics [12]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

same cell culture type under conditions in which TrxRFP1 shows that Trx is significantly oxidized, then a testable hypothesis is that the flux of the thioredoxin system should also be high as measured by RX1.

Declaration of competing interest

I have no conflict of interest.

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