ANTIOXIDANTS & REDOX SIGNALING
Volume 16, Number 7, 2012

DOI: 10.1089/ars.2011.4251

FORUM REVIEW ARTICLE

Assessment of Physiological Redox State with Novel FRET Protein Probes

Masahide Oku[1] and Yasuyoshi Sakai[1–3]

Abstract

Significance: Development of redox-sensing fluorescent proteins (redox probe proteins) have enabled live imaging of the physiological redox state within a cell, generating new strategies for detecting changes in the redox state during developmental, pathogenic, and aging processes. Several of the probe proteins utilize their characteristic redox-sensing segments as linkers in between two fluorophores, where structural alternations of the segments lead to changes in FRET efficiencies across the fluorophores. In this review we summarize two types of novel FRET-based redox probe proteins, namely redox linker (RL)-derived probes and Redoxfluor. ***Recent Advances:*** After these FRET-based redox probe proteins were generated, their responsiveness toward redox-related compounds as well as toward reactive oxygen species or reducing stimuli was investigated *in vitro*. Notably, both the RL-derived probe and Redoxfluor were found to directly respond to the redox state of glutathione, a main redox-formulating compound, showing a promising property for their use in subsequent *in vivo* analyses. Redoxfluor was not only used for redox sensing in the cytoplasm, but also utilized for assessing the redox state within peroxisomes. ***Critical Issues:*** In contrast to "one-fluorophore" redox probes such as roGFP and rxYFP proteins, whose usage has been established and widely expanded to various experimental systems, FRET-based redox probes were invented very recently and their applications to *in vivo* studies are still in their infancy. ***Future Directions:*** FRET-based redox probes provide novel approaches for redox sensing that are complementary to other methodologies. *Antioxid. Redox Signal.* 16, 698–704.

Introduction

In attempts to reveal the intracellular redox state, the use of protein probes has several advantages over dye-based probes. While many of the redox-sensing dyes (*e.g.*, dichlorofluorescein and its derivatives) stain the cells in an irreversible manner (33), protein probes whose thiol residues undergo oxidation/reduction cycles are able to elicit reversible responses, which is suitable for long-term observation of the redox state *in vivo*. Moreover, when tagged by organelle localization signals, protein probes can be utilized for redox state monitoring at the organelle level. These features have led to prominent live-cell imaging studies in a variety of biological fields, such as those investigating mitochondrial redox changes in mammalian culture cell lines in the context of pathological analyses (23,34) or visualizing redox transitions inside a plant model organism (*Arabidopsis thaliana*) under developmental or stress-loaded conditions (13,28).

Precise monitoring of the redox state with protein probes requires ratiometric methodology; the signal indicating redox state should be acquired in parallel with a control signal reflecting the protein probe amount, in order not to over- or underestimate the mean signal intensity due to fluctuations of the probe amount. To date, several fluorescent protein probes for the ratiometric analysis of the redox state have been widely utilized, such as HyPer, roGFP, and rxYFP, as well as their derivatives (2,7,27). A common feature of these probes is that they sense redox changes through structural changes within their single fluorophores (core structures responsible for fluorescence emission) leading to their different excitation spectra depending on redox conditions (3,24).

As an alternative strategy to achieve the ratiometric signal acquisition, FRET-based redox probe proteins possessing two intact fluorophores were devised. FRET (fluorescence or Förester resonance energy transfer) is a phenomenon in which the excitation energy held by one fluorescent (donor) molecule is conveyed to another (acceptor) molecule due to the proximity and appropriate orientation of the molecules, which yields fluorescence from the acceptor molecule. Since FRET efficiency is known to be inversely proportional to the sixth power of the distance between the two fluorophores, it is used as a very sensitive index of intra- and intermolecular

[1]Division of Applied Life Sciences, Graduate School of Agriculture, and [2]Research Unit for Physiological Chemistry, the Center for the Promotion of Interdisciplinary Education and Research, Kyoto University, Kyoto, Japan.
[3]CREST, Japan Science and Technology Agency, Tokyo, Japan.

distances in biochemical analyses (15). Furthermore, thanks to the widely used technique of microscopic FRET imaging with commercially available filter sets, FRET probes provide an attractive strategy for visualization of biological activities in living cells (30). The redox-sensing properties of these FRET-based proteins are attributed to structural alternations within their redox-sensing regions (RSRs) inserted between the two fluorophores (Fig. 1). In this review we described two types of FRET-based redox probes with sufficient biochemical characterization: redox-linker (RL)-derived probes and Redoxfluor. The RSRs of RL-derived probes were constructed from specific peptides designed to form alpha-helices under reduced conditions (16,17,22). Redoxfluor utilizes the C-terminal domain of the *Saccharomyces cerevisiae* Yap1 protein (Yap1p) as the RSR (37). This review first describes molecular mechanisms underlying the functions of these RSRs and their biochemical properties in reference to Yap1p or other protein probes, especially to roGFP probes. Subsequently, applications of these probes to *in vivo* analyses are described.

Molecular Architecture of RL-Derived Probe Proteins

Structural analysis of peptides with repeats of EAAAK amino acid residues demonstrated that they form alpha helices and can be used as linkers for FRET probes (1). Cysteine residues are inserted into these peptides for the purpose of introducing "disulfide turns" in the peptide structure under oxidized conditions. Various combinations of the cysteine residue insertions were made to the peptide containing five repeats of the EAAAK, and one modified peptide termed RL5 was found to confer the sharpest responsiveness of FRET efficiency between the fluorophores connected at both ends, depending on oxidized/reduced conditions (17). Based on this RL5 peptide, a more extended version of the cysteine-inserted peptide, termed RL7, was constructed and used for the linker between a CFP-YFP couple or their derivative fluorophores. This probe showed a remarkable decrease in the FRET yield under reduced conditions, whereas it retained high FRET efficiency under oxidized conditions as seen in the original RL5 case, thus providing an ideal FRET-based redox probe (16). Hereafter are described the biochemical properties and examples of *in vivo* application in terms of this RL7-derived probe, as a representative RL-derived probe.

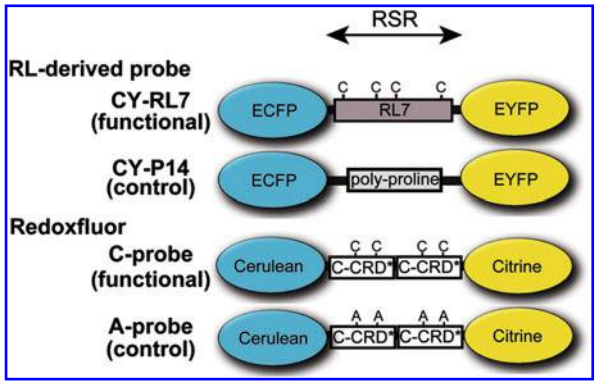

FIG. 1. Schematic drawing of functional and control FRET-based redox probe proteins. The redox-sensing region (RSR) of the two probe proteins are depicted as bars between the two fluorophores. RL7 is based on repeated helical linker sequences intercalated with four cysteine residues. C-CRD* represents the partial domain of Yap1p C-CRD excluding the cysteine 598 residue (amino acids 601–650). In addition to the functional probe proteins, their control (counterpart) constructs are also shown. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

Molecular Mechanism of Yap1p Response and Its Application to Redoxfluor Development

The yeast anti-oxidative responses are regulated by several transcription factors including Yap1p. As a DNA binding protein, Yap1p has a basic leucine zipper (bZIP) domain in its N-terminal region. The transcriptional activity of Yap1p is regulated by its localization changes between the cytoplasm and the nucleus. This protein contains both nuclear localization signal (NLS) and nuclear export signal (NES). Various oxidative stimuli induce conformational changes of Yap1p as described below, which lead to masking of NES and dominant localization of the protein in the nucleus (21,36).

The regulatory region of Yap1p (excluding the NLS and bZIP regions) has six cysteine residues—composing the cysteine-rich domain (CRD)—which are located either in the N-terminal (C303, 310, and 315) or C-terminal (C598, 620, and 629) regions, designated N-CRD or C-CRD, respectively (Fig. 2). Since the C-CRD overlaps with the NES region, structural changes involving C-CRD determine the localization of Yap1p. Among reactive oxygen species (ROS) molecules that affect Yap1 localization, H_2O_2 or tert-butyl hydroperoxide (t-BOOH) induce the formation of disulfide bonds between the cysteine residue in N-CRD (C303) and one in C-CRD (C598) (6). This disulfide bond formation is dependent on another protein termed Gpx3 (or Orp1) protein. The oxidized Gpx3p interacts with Yap1p, which leads to disulfide bond formation across the CRDs. In addition, two interdomain disulfide bonds (C310-C629 and C315–620) that contribute to the enhancement and persistence of Yap1p action are also formed in response to H_2O_2 (25).

In contrast to the case with H_2O_2 treatment, Yap1p response to a thiol-specific oxidant diamide does not rely on disulfide bond formation across the CRDs, but is dependent on the bond formation linking the cysteine residues within the C-CRD (5,20). Thus Yap1p senses various sorts of oxidative stresses by forming different types of disulfide bonds. The formed disulfide bonds are cleaved back to reduced residues in exchange for thioredoxin oxidation *in vitro* (20,25). Consistent with this property, loss of thioredoxin or thioredoxin reductase was found to cause exaggerated localization of Yap1p in the nucleus, indicating excessive oxidation of Yap1p under these conditions (12).

After screening various constructs containing the Yap1p CRD parts, a tandem repeat of a partial region within the C-CRD was found to serve as a good RSR for Redoxfluor (Fig. 2). In parallel with the probe harboring this functional RSR (termed C-probe), a derivative form of Redoxfluor was also constructed in which all of the cysteine residues in the RSR were replaced by alanine (termed A-probe) (Fig. 1).

Responses of FRET-Based Probes to ROS or Reducing Reagents

The probes with RL7 peptide were extensively tested for their reactivities toward the reducing reagent, dithiothreitol

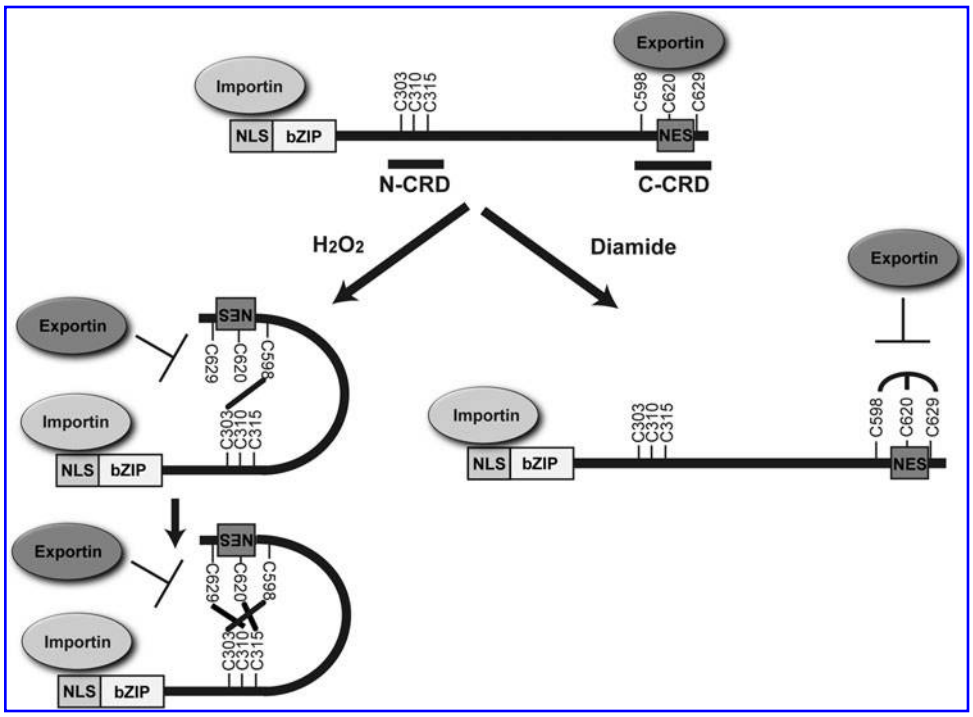

FIG. 2. Conformational changes of Yap1p in response to reactive oxygen species (ROS) molecules. The primary structure of *S. cerevisiae* Yap1p is shown. The pivotal six cysteine residues are also shown as C with the residue numbers. In response to ROS stimuli, disulfide bonds between the cysteine residues (shown as bold lines across the residues) are formed that inhibit Yap1p interaction with Exportin (Crm1p).

(DTT). In the presence of 10 mM DTT, the FRET efficiency of CY-RL7 (RL7 peptide interposed between CFP-YFP couple) dropped to ~17% of that in the original (nonreduced) state, and a comparable decrease in the FRET efficiency was evident even with 1 mM DTT treatment (Fig. 3). These changes of FRET efficiencies are consistent with the design of the RL-derived probes: the more their RSRs are reduced, the longer the helical structures of the RL peptides become, leading to a lower FRET efficiency. As a negative control of this response, a polyproline linker was inserted between the CFP-YFP couple; this construct did not show any detectable changes in FRET efficiency under the same conditions.

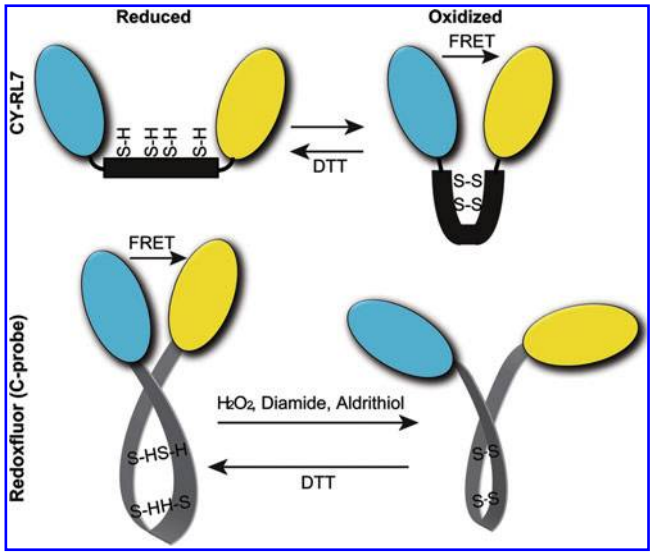

FIG. 3. Schematic model for structural changes of FRET-based redox probe proteins in response to ROS or dithiothreitol (DTT). The fluorophores are represented as ellipses similar to Fig. 1. The thiol or disulfide bonds as well as the reagents responsible for the conformational changes are also shown. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

In contrast, purified Redoxfluor responded to several ROS-generating stimuli and showed decreased FRET efficiency in a biochemical analysis (Fig. 3). The reagents acting on Redoxfluor include H_2O_2, diamide, and aldrithiol, but t-BOOH did not affect the FRET efficiency. The negative-control version of Redoxfluor (A-probe), in which all cysteine residues within its RSR were mutated to alanine, did not change its FRET efficiency following treatment with any of the reagents described above. Conversely, Redoxfluor was found to have increased FRET efficiency following treatment with 1 mM DTT (Oku et al., unpublished data).

The reversed responses to the redox changes by Redoxfluor (with FRET efficiency decreased by oxidation), compared to those by the RL-derived redox probes (with FRET efficiency decreased by reduction), may be attributable to the complicated structure of Redoxfluor RSR (Fig. 1). Based on the structural information of Yap1p CRDs, each of the two c-CRDs in the RSR is predicted to form three alpha helices with two beta sheets positioned in front of the last alpha helix (35). Under oxidized conditions, formation of disulfide bond(s) between the cysteine residues of Redoxfluor RSR might "twist" the structure, detaching the adjacent fluorophores, leading to a decrease in the FRET efficiency (Fig. 3).

CY-RL7 and Redoxfluor Sense Redox Potential of Glutathione

Since glutathione is a major redox-regulating compound in living cells, it is desirable to know the effects of glutathione redox potential on the FRET efficiency of Redoxfluor. Given the equilibrium of glutathione oxidation/reduction as 2GSH↔GSSG (GSH, reduced glutathione; GSSG, oxidized

and thus dimerized glutathione), the redox potential of glutathione is defined as

$$E = E_0 - RT/2F \times \ln(GSH^2/GSSG)$$

(E_0, standard potential for glutathione [−240 mV]; R, gas constant; T, absolute temperature; F, Faraday constant) (19). In order to test Redoxfluor responsiveness to the glutathione redox potential, an incubation buffer system with varying ratios of reduced versus oxidized glutathione was established for the assay of FRET ratio determination. The total concentration of glutathione was adjusted to 2 mM, a value reported to be within the range of its intracellular concentration (24).

Notably, CY-RL7 showed a gradual decrease in FRET efficiency, whereas Redoxfluor (C-probe) showed a gradual increase in efficiency, as the redox potential of glutathione decreased (became more reductive) (Fig. 4). The mutated Redoxfluor (A-probe) did not show significant changes in efficiency under the same conditions. This indicates that RSRs of both CY-RL7 and Redoxfluor changed conformation in accordance with the redox potential of glutathione. The conformational changes occurred without the aid of any redox-related enzymes, in contrast to the case with roGFP derivatives or rxYFP, where efficient reduction of their key residues for the structural changes requires glutaredoxin (glutathione-dependent oxidoreductase) (9).

The midpoint redox potentials sensed with CY-RL7 and Redoxfluor were found to be −143 mV and −213 mV, respectively, by *in vitro* experiments using the reduced/oxidized glutathione couple (Fig. 4). This value is higher (more oxidized) compared with those of roGFP proteins (−299 to −280 mV, in a buffer system containing various ratios of oxidized/reduced DTT) or that of rxYFP protein (−261 mV, in the glutathione buffer) (7,26). The reported intracellular redox potential of glutathione varies from −320 mV to −165 mV depending on the cell type, growth stage, and cell fate (differentiation, apoptosis, *etc.*) (9,14,27). Thus the FRET-based redox probes can be utilized as a means of monitoring cellular redox state complementarily to other redox-monitoring probes whose detectable redox potentials cover more reductive ranges.

Redoxfluor Responses to Thioredoxin

In addition to glutathione, thioredoxin plays an important role in maintaining the redox state in cells. It can directly eliminate some ROS molecules (4) and also can act as the reducing equivalent for activities of peroxiredoxin enzymes (18). Since the RSR of Redoxfluor derives from Yap1p whose activity depends on thioredoxin as described above, the responsiveness of Redoxfluor to the molecule is assumed. Indeed, thioredoxin application at a physiological concentration to oxidized Redoxfluor recovered the FRET efficiency *in vitro* (Oku *et al.*, manuscript in preparation). These responses were not evident with other redox probe proteins; in the case of roGFP2, no significant reduction of this probe was observed even when thioredoxin was directly conjugated to the probe protein to enhance the interaction between these factors (9).

The responsiveness of Redoxfluor to both glutathione and thioredoxin indicates that *in vivo* this probe gives an output reflecting the integrated status of these redox compounds (Fig. 5). Hence it is conceivable that Redoxfluor shows a different response from that of roGFP or rxYFP, which mainly sense the redox state of glutathione (via the activity of glutaredoxin) under physiological conditions. Although both glutathione and thioredoxin rely on NADPH in terms of their recycling from oxidized to reduced states, the recycling steps are catalyzed by distinct enzymes, namely glutathione reductase and thioredoxin reductase. Therefore, the redox state of one molecule *in vivo* may not completely correlate with that of the other one, due to variations in the enzymatic activities (Fig. 5). In this sense, the use of Redoxfluor in parallel with other specific redox indicators is ideal for comprehensive elucidation of the redox state *in vivo*.

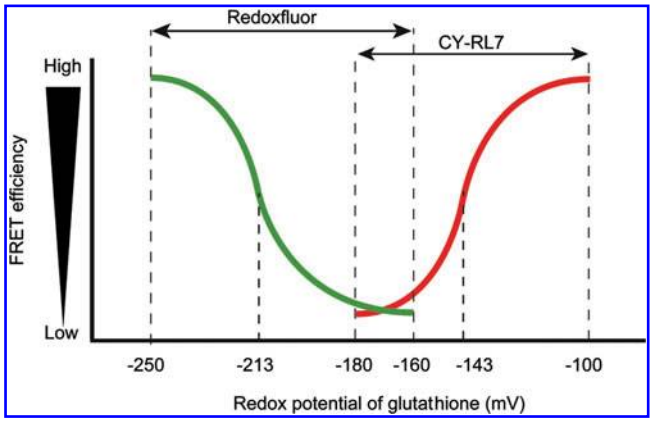

FIG. 4. Schematic comparison of sensing property toward glutathione redox potential between CY-RL7 and Redoxfluor. The distributions of FRET efficiency in the designated redox potentials of glutathione are conceptually drawn in green for Redoxfluor C-probe and in red for CY-RL7. The midpoint potentials calculated in *in vitro* titration analyses are also indicated. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

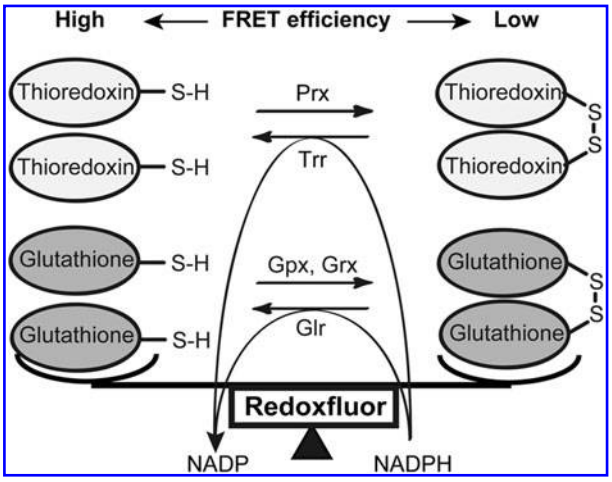

FIG. 5. Drawing of Redoxfluor responses to various redox compounds. Redoxfluor is conceptually represented as a pair of scales monitoring the balance of the redox state. Under physiological conditions, it mainly senses the redox potentials of glutathione and thioredoxin. The reactions affecting the redox state of glutathione and thioredoxin are also shown with abbreviations: Grx, glutaredoxin; Gpx, glutathione peroxidase; Glr, glutathione reductase; Trr, thioredoxin reductase; Prx, peroxiredoxin.

Applications of CY-RL7 to *In Vivo* Analyses

When expressed in Chinese hamster ovary (CHO) cells, the CY-RL7 probe increased its FRET efficiency upon treatment with diamide, and washout of diamide led to a decrease in FRET efficiency back to the original level after a several-minute lag period, indicating the reversible responsiveness of this probe (22). The lag period between the diamide washout and the onset of the drop in FRET efficiency was prolonged when the cells were pretreated with buthionine sulfoximine (BSO; an inhibitor of glutathione synthesis) or bis-chloroethylnitrosourea (BCNU; an inhibitor of glutathione reductase), both of which inhibited the increase in reduced glutathione pool inside cells. This result reflects the responsiveness of CY-RL7 toward the glutathione redox potential that was perturbed by diamide or the inhibitor treatments.

The response of CY-RL7 seen in the diamide pulse experiment was utilized for revealing physiological impacts of the glutathione-related inhibitors on tumorigenic cells (22). The probe was expressed in the porcine tumorigenic cell line 161-T and in the counterpart 161-C (nontumor) cell line. Notably, the increase in the FRET efficiency after the diamide pulse was sustained for more than twice as long as in the 161-T cells under the BSO- or BCNU-pretreated conditions, compared with 161-C under the same conditions, which indicated the prolonged oxidation was occurring in the tumorigenic cell line.

Redoxfluor Responses *In Vivo*

Using fluorescence microscopy, the responses of Redoxfluor expressed in *S. cerevisiae* or CHO cells to various reagents were visualized. This probe had decreased FRET efficiency upon treatment with H_2O_2 or diamide in both experimental systems as *in vitro* responses, and washout of the reagents led to the recovery of the FRET efficiency to the original state, indicating the reversible responsiveness of this probe (similar to CY-RL7). Inhibitors of redox-related enzymes, such as 3-amino 1,2,4,-triazole (a catalase inhibitor) and BSO, did not affect the FRET efficiency of Redoxfluor *in vitro*, but it did decrease the FRET efficiency of the probe in *S. cerevisiae*. This discrepancy is reasonable considering the indirect effects of these inhibitors on cellular redox state: inhibition of the target enzymes leads to a deficiency of the anti-redox system, which in turn causes elevated ROS levels in these cells.

Monitoring of Redox State at the Organelle Level with Redoxfluor

In eukaryotic cells, many of the redox reactions are compartmentalized in organelles such as the mitochondria and peroxisomes, which prevents detrimental diffusion of ROS molecules throughout the cell, as well as improves reaction efficiencies by locally concentrating the enzymes/substrates (8). Detailed analyses of redox states within the organelles, therefore, are required for elucidating the cellular redox regulation system. Owing to advances in protein engineering techniques, the redox-sensing protein probes can now be successfully targeted to mitochondria and used for monitoring the redox states therein (10,29,34).

The peroxisome is one of the main organelles where ROS-producing reactions take place vigorously, *e.g.*, the reaction by acyl-CoA oxidase for beta oxidation of fatty acids that eventually generates H_2O_2 (32). In a methylotrophic yeast species that can grow on methanol as the sole carbon source, alcohol oxidase is localized in the organelle to produce formaldehyde and H_2O_2 (31). Although it is widely known that the production of ROS molecules (including H_2O_2) within the peroxisome is counteracted by anti-oxidative enzymes such as catalase, which is also localized in this organelle, the precise redox state within the organelle as an output of these activities was unclear. In order to monitor the intra-peroxisomal redox state, Redoxfluor was C-terminally tagged with peroxisome targeting signal 1 (PTS1) comprised of three amino acid residues. This tagging did not interfere with the redox-sensing properties of Redoxfluor as judged by its intact responsiveness to H_2O_2. When this organelle-targeted version of Redoxfluor was expressed in CHO cells and in the methylotrophic yeast *Pichia pastoris*, the FRET ratios gained from the probe demonstrated intriguing results (Fig. 6A and B). The peroxisomal Redoxfluor in the CHO cell line under ordinary culture conditions gave a higher FRET ratio than that from the cytoplasmic probe, suggesting a more reductive state inside the peroxisome (Fig. 6A). On the other hand, the intra-peroxisomal probe in the methylotrophic yeast exhibited a higher FRET ratio when the cells were grown on oleate (a beta-oxidation–inducing condition), but much lower ratio when grown on methanol as the carbon source (Fig. 6B). These results indicate the effects of intra-peroxisome metabolism on the redox state within the organelle.

The data obtained from the Redoxfluor study is consistent with the result from a recent report using PTS1-tagged roGFP2 in mammalian cell lines (11). In the roGFP2 experiment, the intra-peroxisomal redox state was slightly more

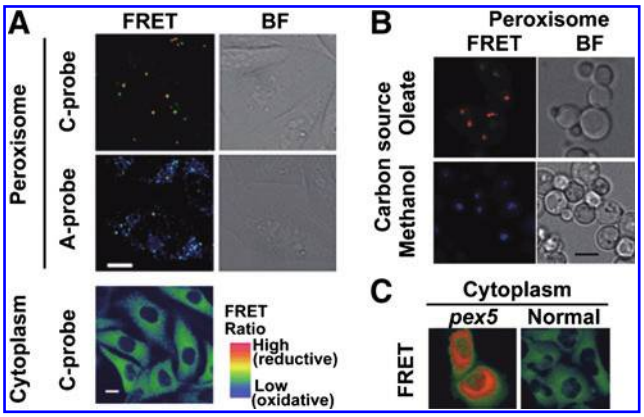

FIG. 6. Redoxfluor application to peroxisome research. (A) Either the peroxisome- or cytoplasm-targeted Redoxfluor was expressed in the CHO cell line, or the FRET ratios (FRET) were visualized according to the color index. The corresponding bright-field images (BF) are also presented for the peroxisome-targeted probe experiment. Bar, 10 μm. **(B)** The peroxisome-targeted Redoxfluor was expressed in the methylotrophic yeast *Pichia pastoris*, and the FRET ratio was visualized as in (A) after the cells were grown on oleate or methanol as a sole carbon source. Bar, 2 μm. **(C)** The cytoplasmic Redoxfluor was expressed in a *pex5* mutant and in normal CHO cells, and its FRET ratio was visualized. The figures were reconstituted from (37).

oxidized than the cytoplasmic state when the cells were cultured in medium supplemented with ascorbic acid, but the tendency was reversed when the cells were cultured in medium without ascorbic-acid supplement (the same conditions as the Redoxfluor experiment). Together, these results indicate that multiple factors can affect the redox state within the organelle.

Another experiment with Redoxfluor demonstrated an unexpected link between peroxisome assembly and redox state. The FRET ratio gained from the cytoplasmic Redoxfluor in a *pex5* mutant strain (deficient in protein transport to the peroxisome) was higher than that in the wild-type strain, indicating a more reductive state of the cytoplasm in the mutant (Fig. 6C). To date, the molecular mechanism of how peroxisome dysfunction causes cytoplasmic reduction has not been elucidated. However, the use of Redoxfluor led to the identification of a "redox modulator" compound that narrows the gap in the redox state between the mutant and wild-type strain (37).

Future Perspectives

Since the FRET-based redox probes are functionally divided into distinct segments, namely RSR and fluorophores, improvement of their responsiveness to various redox stimuli will be accomplished by further modifications within each of the segments. In particular, employing domains of other redox-sensing proteins as RSRs is a plausible strategy to generate a new redox probes.

The multiple responsiveness of Redoxfluor enables us to detect quantitative changes of various redox-related compounds (ROS molecules, glutathione, and thioredoxin) *in vivo*, but at the same time, the factors influencing the FRET efficiencies of Redoxfluor should be identified by other methodologies. Thus the combination of the Redoxfluor technique with a metabolomic analysis or a biochemical determination of each redox compound is ideal for the thorough examination of the redox state. One remarkable feature of Redoxfluor utilization is that this technique allows us to carry out high-throughput analyses that would be difficult with other methods. Therefore, the Redoxfluor technique can be applied to screening experiments for mutants or chemical compounds that affect redox state.

Regarding the monitoring of redox state at the organelle level, the use of FRET-based probes for sensing the intramitochondrial redox state is anticipated for pursuing the function of this vital organelle. Such studies have already been conducted using roGFP protein derivatives (10,29,34), but taking into account the functional differences between the FRET-based probes and roGFP proteins, especially in terms of the responsiveness to thioredoxin, the results from Redoxfluor experiments will be of value for obtaining more detailed information on the redox regulation inside the organelle.

Acknowledgments

We thank Dr. Hiroya Yurimoto and Dr. Jun Hoseki for their valuable discussions. This work was supported by a CREST grant from the Science and Technology Agency of Japan, Grant-in-Aid for Scientific Research 22380502 from Japan Society for the Promotion of Science, and a research grant from The Uehara Memorial Foundation, Japan.

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Address correspondence to:
Dr. Yasuyoshi Sakai
Division of Applied Life Sciences
Graduate School of Agriculture
Kyoto University
Kitashirakawa-Oiwake, Sakyo
Kyoto 606-8502
Japan

E-mail: ysakai@kais.kyoto-u.ac.jp

Date of first submission to ARS Central, August 24, 2011; date of acceptance, September 1, 2011.

Abbreviations Used

BCNU = bis-chloroethyl-nitrosourea
BSO = buthionine sulfoximine
bZIP = basic leucine zipper
CHO = Chinese Hamster Ovary
CRD = cysteine-rich domain
DTT = dithiothreitol
FRET = fluorescence (or Förester) resonance energy transfer
NES = nuclear export signal
NLS = nuclear localization signal
PTS = peroxisome targeting signal
RL = redox linker
ROS = reactive oxygen species
RSR = redox-sensing region
t-BOOH = tert-butyl hydroperoxide
Yap1p = *Saccharomyces cerevisiae* Yap1 protein

This article has been cited by:

1. Marc Fransen, Marcus Nordgren, Bo Wang, Oksana Apanasets. 2012. Role of peroxisomes in ROS/RNS-metabolism: Implications for human disease. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1822**:9, 1363-1373. [CrossRef]