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In situ visualization and detection of protein sulfenylation responses in living cells through a dimedone-based fluorescent probe†

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Sulfenylation is one of the reversible post-translational modifications, playing significant roles in cellular redox homeostasis and signaling systems. Herein, small fluorescent probe (**CPD** and **CPDDM**) based live-cell labelling technology for the visualization of protein sulfenylation responses in living cells has been developed. This approach enables the detection of protein sulfenylation without the need for cell lysis, fixation or purification, and permits the noninvasive study of protein sulfenylation in live cells through the direct fluorescent readout. This technology also can realize dynamic tracking of protein sulfenylation *in situ* with minimal perturbation to sulfenylated proteins and less interference with cellular function. Information on the global distribution and dynamic changes of endogenous protein sulfenylation has been obtained.

Introduction

The homeostasis of the cellular redox environment is one of the most important foundations of living systems. Among the important factors associated with redox homeostasis and signaling, protein thiol is especially attractive for its direct involvement in many biological processes through post-translational modification.[1] Sulfenylation, *i.e.* reversible oxidation of protein thiols into sulfenic acid (R-SOH), has received tremendous attention in recent years.[2] It was initially found to regulate the activity of an important metabolic enzyme, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), in 1969.[3] It has been proven now that sulfenylation is one of the most direct and reversible post-translational modifications in the cell antioxidant defense system,[4] and has significant roles as the intermediate during enzyme catalytic cycles,[3] the regulator of protein translocation,[5] the modulator of gene expression[6] and the global mechanism for cell signaling.[7] Protein sulfenic acid is a highly reactive intermediate because it can be reduced to thiol quickly, or undergoes reaction with a nearby thiol to form a disulfide, enzymatically or nonenzymatically.[8] In the presence of strong oxidants, sulfenic acid would be further converted to sulfinic (Cys–SO_2H) and/or sulfonic (Cys–SO_3H) acid derivatives,[9] which are barely reversible.[10] Therefore, sulfenic acid, usually the first product of thiol oxidation, acts as an active intermediate in the complex thiol redox network.[4,8,11]

Due to the significant role and high reactivity of protein sulfenic acid, a variety of approaches have been developed for detecting sulfenic acid in proteins. By monitoring the difference in UV-vis absorption spectra between the adduct of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazol (NBD-Cl) with sulfenic acid and thiol, the sulfenylation of purified protein was able to be detected *in vitro*.[12] The C-terminal cysteine rich domain of the yeast Yap1 transcription factor was engineered to trap the sulfenic acid.[2] More effort was directed towards the specific and versatile chemical approaches.[13] A method with sodium arsenite was used to reveal the proteomic sulfenylation response of cells towards hydrogen peroxide.[14] The dimedone (5,5-dimethyl-1,3-cyclohexanedione) and its derivatives were the popular ligands for their specific interaction with sulfenic acid.[15] Some mass spectrometry[8,16] and immunochemical approaches[2,17] have been introduced to monitor the formation of sulfenic acids in proteins or even the *in situ* imaging of protein sulfenic acid distribution in fixed cells based on dimedone. The small-molecule probes have also been synthesized through the incorporation of biotin,[18] fluorophore,[2] isotope-coded affinity tags[2,19] or series of azide-based tags[2,20] into

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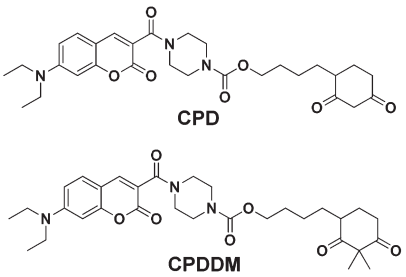

Scheme 1 Structures of **CPD** and **CPDDM**.

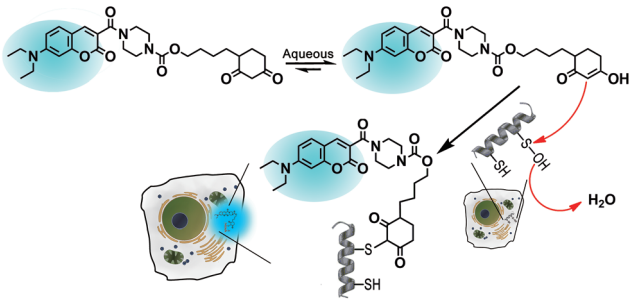

Fig. 1 Plausible mechanism for the specific detection of sulfenic acid moieties in proteins with **CPD**.

dimedone-like derivatives, as well as the 1,3-cyclopentanedione[21] and β-ketoesters,[22] which can selectively label sulfenic acid in protein and make proteomic analysis of protein sulfenylation possible in biological media. Despite this progress, as sulfenic acid is a highly reactive intermediate, some time-consuming procedures with cell lysis, fixation or purification might make the data interpretation complicated and difficult. Furthermore, to investigate the dynamic and compartmentalization of cellular protein in different thiol oxidation states, these kinds of thiol should be specifically labeled and imaged, ideally in living cells.[23] We previously developed *in situ* chemical staining methods for *S*-nitrosothiols and disulfides in fixed cells, imaged their intracellular distribution and observed the central role of mitochondria produced oxygen species in the oxidative modification of thiols.[24] Hence a noninvasive and versatile approach with *in situ* labeling and imaging in living cells is still an urgent need for investigating the dynamic and compartmentalization of cellular protein sulfenylation.

In this study, we reported the visualization of protein sulfenylation in living cells using a dimedone-based fluorescent probe (**CPD**, Scheme 1). This small fluorescent probe based live-cell-labeling technology ensured more rapid detection of protein sulfenylation through direct fluorescent readout, avoiding the time consuming nature of cell lysis, fixation or purification. Additionally, **CPD** with low concentration (5 μM) used in labeling, minimized perturbation to sulfenylated proteins and less interference with cellular ROS. Therefore, this noninvasive labeling protocol enabled us to visualize cellular sulfenylated proteins in the native state aimed at studying their global distribution and dynamic changes.

Results and discussion

To visualize protein sulfenylation in living cells, the probe must be selective, stable, water-soluble and cell permeable.[25] As the sulfenic acid is an unstable transient intermediate during the oxidation processes in living cells, it should be detected rapidly and non-invasively with a small molecular fluorescent probe with low steric bulkiness and fast labeling kinetics,[26] which can track and visualize the protein sulfenylation directly. The sulfenic acid reacts with dimedone and its derivatives to form a stable thioether derivative,[2,15] whereas free thiol has no reactivity towards dimedone under the physiological conditions (Fig. 1). Thus it is possible to selectively discriminate sulfenic acid from other forms of thiols in proteins.[2] In this research, the fluorescent probe **CPD** was designed and synthesized through the conjugation of dimedone analogue with a 7-aminocoumarin fluorophore, which had a suitable excitation wavelength, stable fluorescence signal[27] and moderate quantum yield (Φ_F) under the physiological conditions (Table 1). In order to tune the biocompatibility of **CPD**, the piperazine and urethane group (Scheme 1) was introduced as the linker. A control compound (**CPDDM**, Scheme 1) with dimethylated dimedone to block its reactivity towards sulfenic acids was synthesized. The partition coefficient (P) assay showed that the log P value of **CPD** was 1.49 ± 0.16, and that of **CPDDM** was 1.95 ± 0.11 (Table 1), indicating that they were cell-permeable probes and soluble in PBS buffer (pH 7.4, 1% DMSO as the co-solvent).

We then investigated the spectroscopic characteristics of **CPD** and **CPDDM**. The UV absorption and fluorescence intensity of **CPD** displayed negligible changes at pH 7.0–10 (ESI Fig. 1a and b†). The UV absorption and fluorescence spectra of **CPDDM** were almost identical to those of **CPD** in PBS buffer (Fig. 2a). Because protein sulfenylation is related to the redox environment of living cells, we next investigated the effect of redox agents on the fluorescence intensity of **CPD**. As shown in Fig. 2b, little fluorescence changes were observed in the presence of different concentrations of dithiothreitol (DTT)

Table 1 Spectral properties of **CPD** and **CPDDM** in PBS[a]

Solvent	Compounds	$\lambda_{ex,\ max}$ (nm)	$\lambda_{em,\ max}$ (nm)	Stokes shift	ε (M^{-1} cm^{-1})	Φ_F	Log P
PBS	**CPD**	415 ± 2	485 ± 3	70	24 800	0.11	1.49 ± 0.16
	CPDDM	416 ± 2	483 ± 3	67	23 000	0.10	1.95 ± 0.11

[a] *N*-Butyl-4-butylamino-1,8-naphthalimide was taken as the standard for Φ_F.

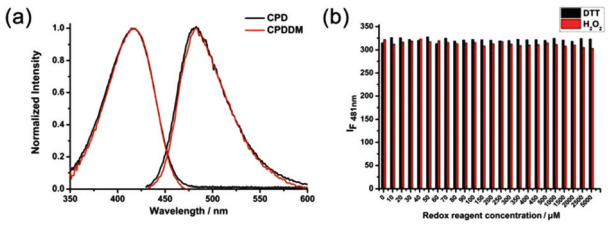

Fig. 2 The spectroscopic characteristics of **CPD** and **CPDDM**. (a) Normalized absorption and fluorescence of **CPD** and **CPDDM** in PBS buffer. (b) Fluorescence responses of 5 μM **CPD** to different concentrations of redox regulating reagents DTT and H_2O_2 (0–5 mM).

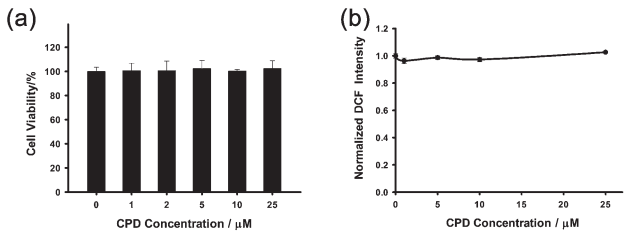

Fig. 3 **CPD** do not interfere with cell viability and the intracellular ROS level. (a) Cell viability test of **CPD**. (b) Effect of **CPD** on cellular ROS detected by 5 μM H_2DCFDA.

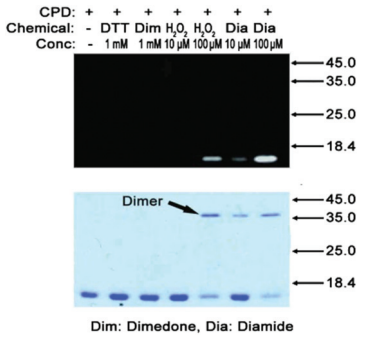

Fig. 4 Selectivity of **CPD** as shown by SDS-PAGE. The labeling of proteins by **CPD** occurred only in the presence of hydrogen peroxide or *N*,*N*,*N*′,*N*′-tetramethylazodicarboxamide (TMAD). DTT: dithiothreitol, Dim: dimedone, H_2O_2: hydrogen peroxide, Dia: TMAD. "+": the molecule was present in the labeling system; "−": the molecule was not present in the labeling system.

and hydrogen peroxide (H_2O_2), which confirmed that **CPD** exhibited stable fluorescence characteristics in various redox environments.

Next, cell cytotoxicity experiments were conducted. **CPD** showed minimal cytotoxicity as shown by the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega) (Fig. 3a). As was reported in the literature,[22,28] high concentration of dimedone (millimolar level) perturbed the cellular redox equilibrium, which may be due to the formation of 4-peroxydimedone radical.[29] In contrast, **CPD** in the micromolar range showed a negligible effect on the intracellular ROS level and cellular redox balance (Fig. 3b). This was also consistent with reported results.[22] In subsequent experiments, 5 μM **CPD** was used for live cell labeling.

Then the specific assay of **CPD** for sulfenic acid was also tested. We used a mono-thiol mutant of thioredoxin (Trx-M, human thioredoxin-2 with C69G mutation, the detailed amino acid sequence in ESI†) as a model protein for *in vitro* sulfenylation labeling. The electrophoresis result demonstrated that **CPD** could label sulfenylated protein selectively in the presence of hydrogen peroxide or TMAD, both of which were able to oxidize thiols to sulfenic acids (Fig. 4). Interestingly, **CPD** did not stain the Trx-M dimer, which was also formed under these oxidative conditions and contained intersubunit disulfide bonds. These *in vitro* labeling data confirmed the selectivity of **CPD** to sulfenic acid in proteins.

Imaging of protein sulfenic acid with **CPD** was tested in Chang liver cells. Marked fluorescence signal was observed in cells stained with **CPD** (Fig. 5a), but none for cells stained with cell permeable **CPDDM** (Fig. 5b and 5f), suggesting the specific labeling of endogenous sulfenylated proteins by **CPD** in living cells. In order to investigate whether the diethylaminocoumarin fluorophore and piperazine alkyl ether linker unit induce a significant bias in probe localization and protein labeling, the **CPD** was introduced into the labeling system before washing. Both **CPD** and **CPDDM** penetrated into Chang liver cells, and showed a similar and wide pattern in cells (ESI Fig. 4a and c†). This result suggested that **CPD** had no bias. After the cells were washed to remove free probes, the **CPDDM** labeled cells had a very weak signal left (ESI Fig. 4c,d and Fig. 5b,f†). In contrast, marked fluorescence signal was still observed in cells stained with **CPD** (ESI Fig. 4a,b and Fig. 5a, f†), which further confirmed that **CPD** has no bias and the labeled fluorescence signal was mainly from endogenous sulfenic acids in proteins. We then tracked the dynamic changes of protein sulfenic acids by using **CPD** based live-cell-labeling technology. The fluorescence intensity of **CPD**-labeled cellular sulfenylated protein decreased notably with increasing amounts of dimedone (Fig. 5c and g), owing to the competitive binding of dimedone with the *de novo* formed sulfenic acid in the cells. Co-incubation of the cells with DTT, which could reduce the sulfenic acids to thiols, also decreased the fluorescent staining signal by **CPD** (Fig. 5d, h and i). On the other hand, oxidants such as H_2O_2 and TMAD significantly enhanced **CPD** staining in a dose-dependent manner, by oxidizing free thiols and increasing the cellular protein sulfenylation level (Fig. 5e, j, k, l and m). In these cases, the newly formed sulfenic acid was trapped and labeled by **CPD** upon oxidation (Fig. 5k and m). The increased **CPD** staining under oxidative conditions disappeared when dimedone was present, due to the competitive binding of sulfenic acid by **CPD** and dimedone (Fig. 5j). We also evaluated protein sulfenylation in HL60 cells, the human promyelocytic leukemia cells in suspension (ESI Fig. 5†). It was interesting that TMAD was much more efficient than H_2O_2 to promote sulfenic acid formation in the HL60 cells, while these two oxidants induced similar sulfenylation levels in Chang liver cells, suggesting different redox environments in different cell lines. All these results demonstrated that the cell-permeable **CPD** could be used for imaging and tracking endogenous sulfenylated proteins by the

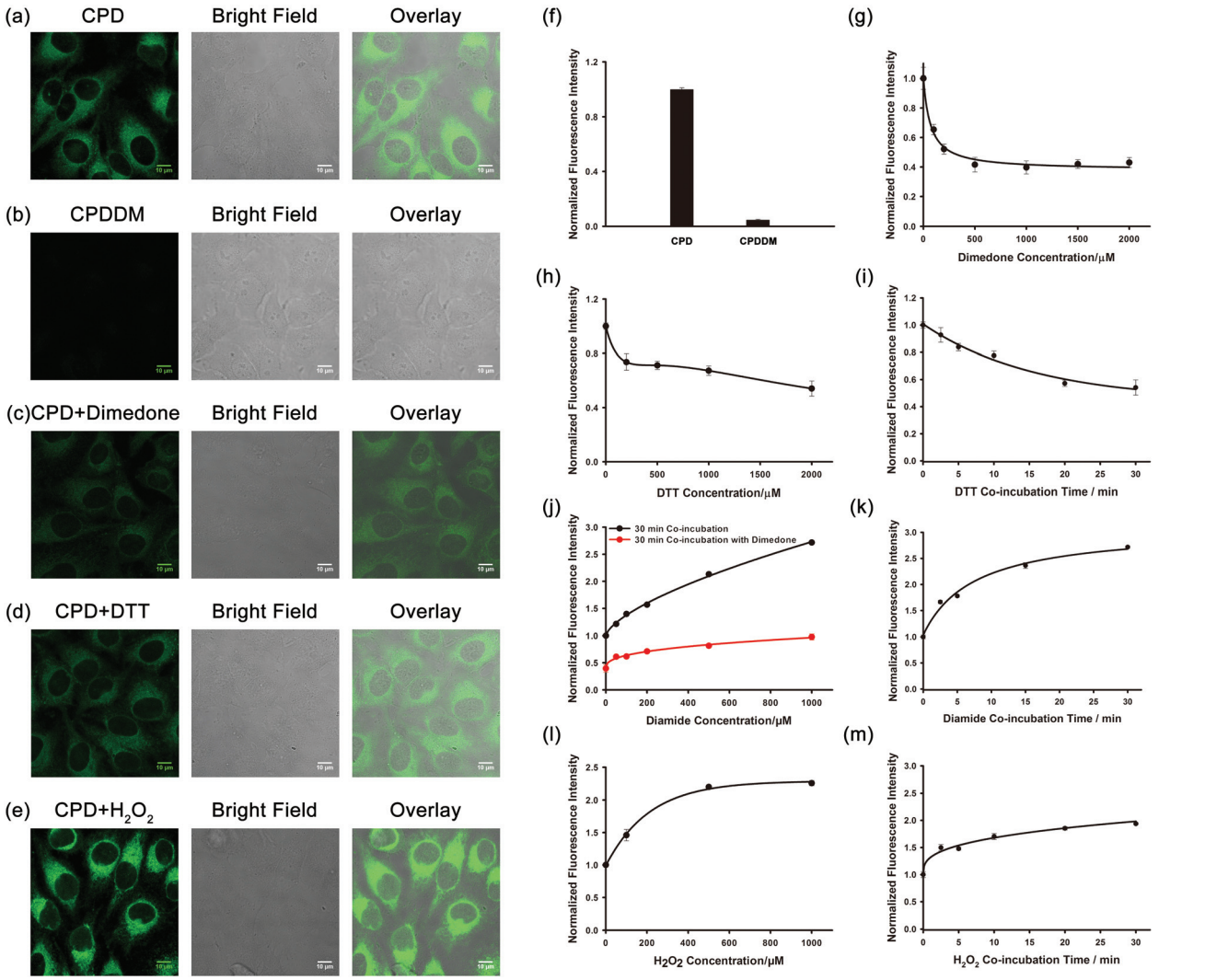

Fig. 5 Live cell imaging of protein sulfenylation in Chang liver cells responding to redox regulations. (a–e) Fluorescent images of cells labeled with **CPD** (5 μM, a), **CPDDM** (5 μM, b), the coumarin fluorophore, **CPD** (5 μM) co-labeled with 1 mM dimedone (c), **CPD** (5 μM) co-labeled with 2 mM DTT (d), and **CPD** (5 μM) labeled in the presence of 1 mM H_2O_2 (e). Cellular responses of **CPD** labeled sulfenylated protein in Chang liver cells to redox regulation (f–m). (f) The fluorescence intensity of **CPD/CPDDM** labeled cells. (g) Effect of different concentrations of dimedone (co-incubation). (h) Effect of different concentrations of DTT. (i) Kinetics of 2 mM DTT reduction (co-incubation). (j) Effect of different concentrations of TMAD on protein sulfenylation. (Black: co-incubated with **CPD**, red: co-incubated with **CPD** and 1 mM dimedone). (k) Kinetics of 1 mM TMAD oxidation (co-incubation). (l) Effect of different concentrations of H_2O_2 (co-incubation). (m) Kinetics of 1 mM H_2O_2 reduction (co-incubation). Error bars represent ±S.E.M. of six measurements.

variation of fluorescence intensity in living cells, and protein sulfenic acid in different cells had different responses toward oxidative conditions.

We next investigated the localization of endogenous protein sulfenic acids by confocal microscopy and co-staining with organelle-specific labels, such as the Mito-Tracker Deep Red staining for mitochondria and DsRed-2 fluorescent protein targeted to ER. The fluorescence signal of the **CPD**-labeled sulfenylated proteins showed mainly a smooth perinuclear and peripheral pattern of a lace-like network, suggesting mainly the endoplasmic reticulum (ER) location (Fig. 5a and 6c). There was a weak signal indicated by the punctuated pattern in the perinuclear zone suggesting mitochondria staining (Fig. 5a). The merged image of **CPD** and mitochondria showed scattered yellow fluorescence (Fig. 6a). Under the oxidative conditions, the signal of **CPD** staining turned out to be much brighter in the focused eccentric perinuclear zone where mitochondria were located (Fig. 6b) with no obvious fluorescent change of the network pattern (Fig. 6d), showing the increase of sulfenylated proteins within mitochondria during the oxidation. These results suggested that the protein sulfenylation occurred mainly in the ER and mitochondria, and the thiols in mitochondria proteins were significantly oxidized to sulfenic acid under the oxidative stress.

In recent years, different detection methodologies to protein sulfenylation have been highlighted. Poole *et al.* synthesized fluorescent or biotinylated derivatives of dimedone.[2] Carroll *et al.* designed a series of azide-based analogs of

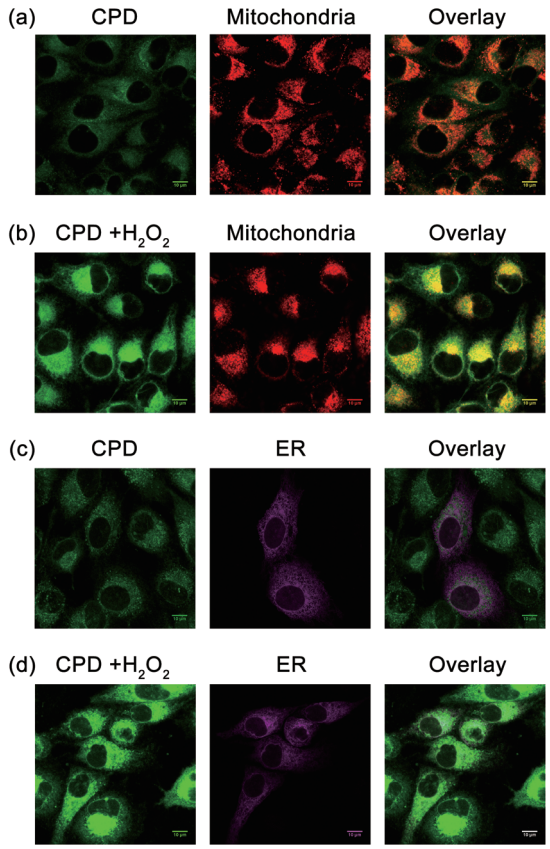

Fig. 6 Co-localization of sulfenylated proteins with mitochondria and ER. (a) Co-localization of sulfenylated proteins labeled with **CPD** with mitochondria labeled with Mito-Tracker Deep Red. (b) Co-localization of sulfenylated proteins trapped by **CPD** during 1 mM H_2O_2 co-incubation with mitochondria labeled with Mito-Tracker Deep Red. (c) Co-localization of sulfenylated proteins labeled with **CPD** with ER-targeted DsRed-2. (d) Co-localization of sulfenylated proteins trapped by **CPD** during 1 mM H_2O_2 co-incubation with ER-targeted DsRed-2.

dimedone,[2,20] and combined the usage of isotope-coded dimedone and iododimedone[2] to quantify the sulfenic acid in proteins. Furthermore, they also developed an immunohistochemistry approach[2,7] for differential analysis of samples with various protein sulfenylation levels by using specific antibodies that identify the epitope conjugated by protein sulfenic acid and dimedone. Herein, we focused on the development of new live-cell-labeling technology by using a small-molecule fluorescent probe. This noninvasive method can be applied to monitor the protein sulfenylation in living cells through direct readout of **CPD** fluorescent staining signal with minimal perturbation to sulfenylated proteins and less interference with cellular function.

By using this versatile live-cell-labeling technology, we found that the basal level of protein sulfenylation already existed in resting cells, suggesting that it was an important physiological regulator of protein activity and the global signaling mechanism,[7] rather than just defense against ROS stress. In resting cells, protein sulfenylation appeared to be abundant in ER as demonstrated by **CPD** labeling of living cells. In a recent work in which protein sulfenylation was detected by dimedone specific antibodies,[2] the immuno-fluorescence signal was shown to be located in ER as well. Protein sulfenylation could happen spontaneously and nonenzymatically during protein folding processes in aqueous solutions containing dissolved oxygen.[8] As the main location for protein synthesis and oxidative folding, ER maintains the most oxidative status in the cell.[29] Therefore, sulfenic acid could be an important intermediate during protein disulfide bond formation in ER. It was interesting that sulfenic acids were also found to be enriched in mitochondria, particularly under oxidative conditions. As the key organelle for respiration, mitochondria are the major locations for many redox-related biochemical processes. We previously reported that in mammalian cells, mitochondria maintained a substantial level of disulfide proteome under resting conditions, which markedly increased upon oxidative stimulation.[24] Similarly, *S*-nitrosoproteins also mainly existed in the mitochondria and perimitochondrial compartment.[24] Protein vicinal dithiols, the reduced end of the thiol redox form, were also found to be concentrated in mitochondria.[30] Furthermore, both *S*-nitrosothiol and disulfide formation were determined by mitochondrial respiration and the generation of reactive oxygen species. All these results suggested that mitochondria were the central participants in thiol redox regulation and might have profound effects on cellular protein function.

Conclusions

In summary, we reported the *in situ* visualization and detection of protein sulfenylation responses in living cells through the direct fluorescence readout with dimedone-based fluorescent probes **CPD**. The control probe **CPDDM** labeled cells displayed no fluorescence signal and further demonstrated that **CPD** could be applied for dynamic tracking of protein sulfenylation without bias. By using this live-cell-labeling technology, we could track dynamic changes of protein sulfenylation and obtained more information about the subcellular distribution of protein sulfenic acid *in situ*, as well as its potential roles in cellular function.

Experimental

Synthesis of CPD and CPDDM

4-(2,4-Dioxocyclohexyl)butyl-4-(7-(diethylamino)-2-oxo-2*H*-chromene-3-carbonyl)piperazine-1-carboxylate (CPD). To a solution of CSO1 (66 mg, 0.12 mmol) in THF–DCM (3/1, v/v, 4 mL) was added 4 N HCl (4 mL) in an ice bath. The mixture was stirred at room temperature for about 4 h until TLC shows that the material CSO1 disappeared. Then the reaction mixture was diluted with water (5 mL) and extracted with DCM (3 × 15 mL). The organic phases were combined and dried with anhydrous $MgSO_4$. The organic phase was reduced to dryness and the crude product was purified by column chromatography on silica gel (with DCM–EtOAc–MeOH,

16 : 4 : 1) to obtain a yellow solid (43 mg, 70%). Melting point (mp) 130–135 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.89 (s, 1H), 7.27 (d, 1H, J = 9.2 Hz), 6.62 (dd, 1H, J = 8.8, 2.0 Hz), 6.49 (d, 1H, J = 1.6 Hz), 4.14 (t, 2H, J = 6.4 Hz), 3.74 (br, 2H), 3.58 (br, 4H), 3.48–3.39 (m, 8H), 2.75–1.90 (m, 6H), 1.72–1.40 (m, 5H), 1.24 (t, 6H, J = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 204.4, 203.8, 165.3, 159.1, 157.3, 155.4, 151.8, 129.9, 115.7, 109.4, 107.7, 104.5, 96.9, 65.3, 58.2, 49.2, 44.9, 39.6, 29.6, 29.0, 28.8, 24.5, 23.5, 12.4; IR (KBr, cm^{-1}): 3475, 2925, 2850, 1710, 1620, 1595, 1518, 1456, 1412, 1354, 1231, 1190, 1125, 1077; HRMS (ESI+) calcd for $C_{29}H_{38}N_3O_7$ $[M + H]^+$ 540.2710, found 540.2703; HPLC t_R = 25.20 min (purity >95%, monitored both at 254 and 415 nm).

4-(3,3-Dimethyl-2,4-dioxocyclohexyl)butyl 4-(7-(diethylamino)-2-oxo-2*H*-chromene-3-carbonyl)piperazine-1-carboxylate (CPDDM). To a solution of **CPD** (20 mg, 0.04 mmol) in acetone (4 mL) was added potassium carbonate (11 mg, 0.08 mmol). After refluxing for 30 min, CH_3I (14 mg, 0.09 mmol) was added, and then the reaction mixture was continued to reflux for about 12 h. TLC shows that the material **CPD** disappeared, the reaction mixture was cooled to room temperature and chloroform was added, left for 30 min, and filtrated to get a clean solution. After removing the solvent under reduced pressure, a yellow crude solid was obtained. The crude product was purified by column chromatography on silica gel (with DCM–MeOH, from 100 : 1 to 50 : 1) to obtain a pale yellow solid (13 mg, 62%). Melting point (mp) 155–160 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.91 (s, 1H), 7.36 (d, 1H, J = 8.8 Hz), 6.63 (dd, 1H, J = 8.8, 2.4 Hz), 6.49 (d, 1H, J = 2.4 Hz), 4.15 (t, 2H, J = 6.4 Hz), 3.76 (br, 2H), 3.59 (br, 4H), 3.49–3.43 (m, 6H), 2.88–1.89 (m, 6H), 1.71–1.41 (m, 5H), 1.38 (s, 6H), 1.27–1.23 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 210.8, 210.6, 171.3, 159.1, 157.3, 155.4, 151.9, 129.7, 115.9, 109.6, 108.2, 105.3, 97.1, 65.4, 61.0, 46.0, 45.1, 37.0, 29.7, 29.3, 27.2, 25.6, 24.1, 23.6, 22.6, 19.2, 14.1, 12.3; IR (KBr, cm^{-1}): 3462, 2928, 2856, 1700, 1625, 1599, 1517, 1407, 1345, 1247, 1140; HRMS (ESI+) calcd for $C_{31}H_{41}N_3O_7Na$ $[M + Na]^+$ 590.2842, found 590.2842; HPLC t_R = 31.26 min (purity >95%, monitored both at 254 and 415 nm).

Expression and purification of thioredoxin 2 mutant (Trx)

Human thioredoxin 2 (signal peptide removed) gene with an N-terminal hexahistidine tag was constructed on pET-28a vector (Novogen). To obtain a single thioredoxin mutant, Cys69 was mutated to glycine on pET-28a vector. Thioredoxin 2 C69G mutation (Trx-M) protein was expressed in *Escherichia coli* strain BL21 (DE 3). After 4 hours of induction with 0.1 mM IPTG, *E. coli* cells were harvested by centrifugation. Lysis was obtained by ultrasound, and then centrifuged at 10 000*g* for 30 min at 4 °C. Trx-M was purified from the supernatant using affinity chromatography on a Histrap HP column (GE Healthcare) according to the manufacturer's instructions. The buffer for storage was inter-changed with DPBS using an Amicon Ultrafiltration device from Millipore. The purity of the protein was verified by 15% SDS-PAGE to reach 95%. The purified protein was stored at −80 °C until further use.

Preparation of reduced forms of Trx-M

For the single thiol model of Trx-M, 10 mM DTT was used to reduce the cysteine in the protein. After reduction, excess reductant was removed by gel filtration on Sephadex G-25 against PBSE buffer (PBS buffer prepared by DPBS powder, 5 mM EDTA, pH 7.4).

The status of the thiols in model proteins was verified using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) after incubating proteins with an excess of DTNB in PBSE buffer for 30 min in the dark at room temperature. An absorption coefficient at 412 nm of 14.12 mM^{-1} cm^{-1} was used to quantify the 5-thio-3-nitrobenzoate anion, with the absorbance of the DTNB solution and the intrinsic low absorbance of proteins at this wavelength accounted for. The molar ratio of thiol quantified by DTNB and of protein quantified by absorbance at 280 nm was about 0.9–1.1.

SDS-PAGE and fluorescence imaging of gels

The selectivity of **CPD** was verified by 15% SDS-PAGE electrophoresis. Samples were labeled in the PBS buffer at 37 °C for 30 min, with a final concentration of protein at 50 μM, **CPD** at 100 μM and different concentrations of chemicals. After labeling, the samples were desalted using Bio-Rad Micro Bio-Spin columns with Bio-Gel P-6 in SSC buffer (cat. no. 732-6201). Then, the desalted samples were mixed with SDS-PAGE loading buffer without β-mercaptoethanol. Protein concentrations were quantified by the Bradford method. Different volumes of samples were loaded to make sure the same quantity of proteins was loaded. Then, electrophoresis was started immediately. The gel was imaged using the Carestream In Vivo Imaging FX System (excitation: 420 nm, emission: 480 nm). The same gel was also stained using Coomassie brilliant blue (CBB) after the fluorescent image was obtained.

Cell culture

Chang liver and HL60 cells were obtained from the American Type Culture collection. Chang liver/HL60 cells were grown in DMEM (high glucose) medium/RPMI 1640 medium supplemented with 10% FBS. Chang liver cells were typically passaged with a sub-cultivation ratio of 1 : 4 every two days. HL60 cells were typically passaged with a sub-cultivation ratio of 1 : 10 every two days. Cells were incubated in a 5% CO_2 humidified incubator at 37 °C.

Detection of Chang liver cellular protein sulfenylation response to different chemicals

Cells were seeded on a GE 96-well glass matriplate (cat. no. 28-9323-99) the day before detection with about 80% intensity. During detection, the cells were washed twice using DPBS (containing Ca, Mg) buffer at 100 μL per well each time. Then, the cells were co-incubated with 5 μM **CPD** and different concentrations of chemicals (100 μL per well in all) in DPBS (containing 1% DMSO) for 30 min in a 5% CO_2 humidified incubator at 37 °C. After that, the cells were washed for six times using PBS (containing Ca, Mg) buffer at 100 μL per well

each time. Then 100 μL of PBS was added into each well for detection. Then, the fluorescent intensity was quantified using a Biotek Synergy 2 microplate reader with a 420 nm excitation filter (bandpass: 10 nm) and a 485 nm emission filter (bandpass: 20 nm).

In situ imaging

Chang liver cells were labeled with 5 μM **CPD** in DPBS (containing 1% DMSO) at 37 °C. For the H_2O_2, DTT, or dimedone co-incubated sample, 1 mM H_2O_2, 2 mM DTT or 1 mM dimedone was introduced into the labeling system. For the **CPDDM** sample, the same concentration of **CPDDM** was used instead of **CPD**. After 30 min, the cells were washed six times to remove unbounded **CPD** before *in situ* imaging with a Nikon A1R confocal laser scanning microscope using a Plan Apochromat violet corrected (VC) 60× WI (N.A. 1.20; W.D. 0.27) objective, with excitation by a 405 nm laser, and 425–475 nm emission light was collected.

Colocalization

For **CPD** co-localization with Mito-Tracker sample: cells were pre-washed twice, and labeled with 50 nM Mito-Tracker Deep Red for 15 min at first and then with 5 μM **CPD** as described above.

For **CPD** co-localization with ER-tracker sample: pDsRed-2-ER vector was transfected into Chang liver cells using a Calcium Phosphate Cell Transfection Kit (Beyotime, cat. no. C0508) the day before detection. Then, the cells were labeled by 5 μM **CPD** as described above.

To exclude the interference with the fluorescent signal: the excitation of **CPD** was at 405 nm, and 425–475 nm emission light was collected. The excitation of DsRed-2 was at 561 nm, and 570–620 nm emission light was collected. The excitation of Mito-Tracker Deep Red was at 638 nm, and >650 nm emission light was collected.

Detection of the HL60 cellular protein sulfenylation response to different chemicals

2.5×10^7 HL60 cells were centrifuged at 800 rpm for 5 min, and washed twice with PBS buffer. Then the cells were resuspended in 1 mL DPBS buffer containing 5 μM **CPD** and different concentrations of chemicals. After a 30 min incubation in a rotary shaker in a 5% CO_2 humidified incubator, the cells were washed six times with 1 mL DPBS. Then the cells were resuspended in 500 μL of DPBS buffer for Aria I Flow Cytometry detection. The fluorescent signal was obtained through excitation with a 407 nm laser and 430–470 nm emission light was collected. The flow cytometry data were processed using BD FACSDiva and FlowJo software.

Cell viability test

Chang liver cells were seeded on a Coring 96 well plate the day before detection with about 80% intensity. During detection, cells were treated with different concentrations of dimedone or **CPD** as described above, and then the cell viability was tested using a Promega CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay Kit.

H_2DCFDA test

Cells were seeded on a GE 96 well glass matriplate (cat. no. 28-9323-99) the day before detection with about 80% intensity. During detection, cells were labeled with different concentrations of dimedone for 30 min as described before. Then 100 μL of H_2DCFDA (5 μM) in PBS solution was added to each well. After 30 min of incubation and two washes, the fluorescent intensity was measured using a Biotek Synergy 2 Microplate Reader (ex: 485 nm, em: 528 nm (bandpass: 20 nm)).

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