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Research Paper

Redox-sensitive GFP fusions for monitoring the catalytic mechanism and inactivation of peroxiredoxins in living cells

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ABSTRACT

Redox-sensitive green fluorescent protein 2 (roGFP2) is a valuable tool for redox measurements in living cells. Here, we demonstrate that roGFP2 can also be used to gain mechanistic insights into redox catalysis *in vivo*. *In vitro* enzyme properties such as the rate-limiting reduction of wild type and mutant forms of the model peroxiredoxin *Pf*AOP are shown to correlate with the ratiometrically measured degree of oxidation of corresponding roGFP2 fusion proteins. Furthermore, stopped-flow kinetic measurements of the oxidative half-reaction of *Pf*AOP support the interpretation that changes in the roGFP2 signal can be used to map hyperoxidation-based inactivation of the attached peroxidase. Potential future applications of our system include the improvement of redox sensors, the estimation of absolute intracellular peroxide concentrations and the *in vivo* assessment of protein structure-function relationships that cannot easily be addressed with recombinant enzymes, for example, the effect of post-translational protein modifications on enzyme catalysis.

1. Introduction

Genetically encoded fusion constructs between redox enzymes and redox-sensitive fluorescent proteins are commonly used to make non-invasive redox measurements in living cells [1–4]. Fusion constructs between a peroxiredoxin (Prx), which serves as the peroxide sensor moiety, and redox-sensitive green fluorescent protein 2 (roGFP2), which serves as the reporter moiety, have recently been developed and allow real-time monitoring of intracellular hydroperoxide concentrations [4–6]. Here, we asked whether roGFP2 fusion constructs and hydroperoxide challenges can be used to deduce peroxidase properties and mechanisms *in vivo*. In other words, do classic enzyme kinetic parameters of peroxidases affect the roGFP2 readout in a predictable fashion? In particular, we were interested in understanding how roGFP2 readouts are affected by the peroxidase k_{cat}^{app} values and catalytic efficiencies (k_{cat}^{app}/K_m^{app} values reflecting second order rate constants) as well as inactivation kinetics due to hyperoxidation, *i.e.* the sulfinic and sulfonic acid formation of the active site cysteine residue.

To experimentally address these questions, we required a kinetically well-characterized peroxidase isoform and thus chose to use the Prx5-type model enzyme *Pf*AOP from the malaria parasite *Plasmodium falciparum*. *Pf*AOP localizes to the cytosol and plastid of the parasite, is dispensable for asexual blood stage development and accepts a variety of hydroperoxide substrates and electron donors *in vitro* [7–10]. Steady-state kinetics in combination with site-directed mutagenesis, X-ray structures and gel filtration analyses have previously revealed that *Pf*AOP requires only one cysteine residue for catalysis and predominantly forms stable homodimers [10,11]. This is in contrast to GPx3 and typical 2-Cys Prx [12,13], which have been used as ratiometric roGFP2-coupled peroxide sensors [3,5,6]. The latter peroxidases are either incompletely characterized regarding their kinetics or have more complicated reaction mechanisms due to the number of relevant cysteine residues and/or variable quaternary structures.

We have previously characterized the kinetic parameters of gain- and loss-of-function mutants of *Pf*AOP and showed that the enzyme is rapidly inactivated by H_2O_2 but not by *tert*-butyl hydroperoxide

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(*t*BOOH) [10,11]. Mutation of residue Leu^{109}, which is situated at the bottom of the active site between the catalytic (peroxidatic) cysteine residue Cys^{117} and the buried non-catalytic cysteine residue Cys^{143}, affects the catalytic as well as the inactivation properties of *Pf*AOP [11]. For example, compared to recombinant wild type *Pf*AOP, the gain-of-function mutant *Pf*AOP^{L109M} was shown to be less susceptible to H_2O_2-dependent inactivation and to have a 3-fold higher k_{cat}^{app} value for *t*BOOH, a 2.3-fold higher k_{cat}^{app} value for glutaredoxin (Grx) as an electron donor, and a 1.2- to 1.4-fold higher k_{cat}^{app} value for reduced glutathione (GSH) as an electron donor [11]. Furthermore, the k_{cat}^{app}/K_m^{app} values of *Pf*AOP^{L109M} for Grx and GSH increased 2.2- and 12-fold, respectively, whereas the k_{cat}^{app}/K_m^{app} value for tBOOH was similar to the wild type enzyme. In contrast, the loss-of-function mutant *Pf*AOP^{L109A} was shown to be more susceptible to H_2O_2-dependent inactivation, to have 4-fold lower k_{cat}^{app} values for *t*BOOH and GSH, a 7-fold lower k_{cat}^{app}/K_m^{app} value for tBOOH and a 3-fold lower k_{cat}^{app}/K_m^{app} value for GSH. The activating effects for *Pf*AOP^{L109M} depended on the presence of residue Cys^{143}, although its exact role could not be resolved by steady-state kinetics or gel mobility shift assays. Based on our kinetic and structural data, we proposed a model according to which Leu^{109} and Cys^{143} together affect the equilibrium between the fully folded and locally unfolded conformation of *Pf*AOP. Mutation of Leu^{109} to methionine was suggested to stimulate local unfolding of the active site helix α2, thereby preventing hyperoxidation and promoting the probably rate-limiting GSH-dependent reduction of the Cys^{117} sulfenic acid [11]. Here we used our gain- and loss-of-function mutants to compare the oxidative half-reaction using stopped-flow kinetics and to test the suitability of roGFP2 as a mechanistic reporter for redox catalysis inside living cells.

2. Materials and methods

2.1. Materials

H_2O_2, *t*BOOH, peroxynitrite, cumene hydroperoxide and 12(S)-hydroperoxy-5Z,8Z,10E,14Z eicosatetraenoic acid (12(S)HpETE) were purchased from Mallinckrodt Chemicals or Sigma. The concentration of H_2O_2 stock solutions was determined spectrophotometrically at 240 nm ($\varepsilon_{240\ nm}$ = 43.6 M^{-1} cm^{-1}). The peroxynitrite concentration was determined at alkaline pH at 302 nm ($\varepsilon_{302\ nm}$ = 1.67 mM^{-1} cm^{-1}) [14]. Concentrations of cumene hydroperoxide and 12(S)HpETE were calculated considering the manufactures specifications. Diamide, 1,4-dithiothreitol (DTT), diethylenetriaminepentaacetic acid (DTPA) and horseradish peroxidase were purchased from Sigma. Nickel-nitrilotriacetic acid agarose (Ni-NTA) was from Qiagen. HiTrap desalting columns were from Amersham Bioscience. All of the amino acids, glucose and yeast nitrogen base required for Hartwell's Complete (HC) yeast growth medium were purchased from Sigma. Flat-bottom 96 well microplates (product #353219) were from BD Biosciences.

2.2. Cloning of yeast expression vectors

The gene sequence for N-terminally truncated *Pf*AOP$^{\Delta 59}$ without its apicoplast-targeting sequence [7,10] (herein after referred to as *Pf*AOP) was optimized for expression in *Saccharomyces cerevisiae*, synthesized and cloned into a pUC57 vector (Genscript, Piscataway, USA). *PfAOP* was subcloned into *roGFP2-GRX1*/p416TEF [15] using *Eco*RI and *Hin*dIII restriction sites, thereby replacing *GRX1* to generate wild type *roGFP2-PFAOP^{wt}*/p416TEF. A standard site-directed mutagenesis protocol was employed to generate *roGFP2-PFAOP^{L109M}*/p416TEF, *roGFP2-PFAOP^{L109A}*/p416TEF and *roGFP2-PFAOP^{C143S}*/p416TEF. All mutations were confirmed by Sanger sequencing. The codon-optimized gene sequence encoding roGFP2-*Pf*AOP has been deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under accession number MF140392 and is listed in the Supplementary material.

2.3. Transformation, expression and fluorescence measurements in S. cerevisiae

Plasmids p416TEF, *roGFP2*/p416TEF and wild type and mutant forms of *roGFP2-PFAOP*/p416TEF were transformed into yeast strain BY4742 and roGFP2 measurements were conducted as described previously [3,4]. Briefly, liquid cultures were grown to late exponential phase (OD_{600} = 3–4) in HC medium lacking uracil, in order to select for those cells retaining the p416TEF vectors. Cells were harvested by centrifugation for 3 min at 800 × *g* and resuspended in buffer containing 100 mM NaCl, 100 mM sorbitol, 100 mM Tris-HCl, pH 7.4 to a final concentration of 7.5 OD_{600} units/mL. Aliquots of 200 µL of the cell suspension were transferred into the appropriate number of wells of a flat-bottom 96 well microplate. Two additional wells were utilized for controls and were supplemented with either diamide to a final concentration of 20 mM (fully oxidized control) or DTT to a final concentration of 100 mM (fully reduced control). The control wells are required for the determination of the degree of sensor oxidation (OxD). Cells in the experimental wells were treated with twelve increasing concentrations of H_2O_2 or *t*BOOH (10–500 µM) and responses were followed for up to 100 min at 30 °C using a CLARIOstar fluorescence plate reader (BMG Labtech). For each peroxide concentration the OxD was plotted against time. The area under the curve (AUC (OxD × min)) was subsequently calculated in Excel and plotted against the according peroxide concentration in SigmaPlot 13. All data were averaged from triplicate (*t*BOOH treatment) or quadruplicate (H_2O_2 treatment) measurements from independent yeast cultures. Statistical analyses were carried out in SigmaPlot 13 using the One-way ANOVA method.

2.4. Expression and purification of recombinant wild type and mutant PfAOP in Escherichia coli

Recombinant proteins were expressed and purified by affinity-chromatography as described previously [10,11,16]. Briefly, *E. coli* XL1-Blue cells were transformed with plasmid *PFAOP*/pQE30, *PFAOP^{C117S}*/pQE30, *PFAOP^{C143S}*/pQE30 or *PFAOP^{L109M}*/pQE30. Expression was induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside for 4 h at 37 °C. Liquid cultures were harvested by centrifugation for 15 min at 4000 × *g* and 4 °C. The bacteria were resuspended in buffer containing 20 mM imidazole, 300 mM NaCl, 50 mM $Na_xH_yPO_4$, pH 8.0, incubated with lysozyme and disrupted by sonication. Proteins were affinity-purified on Ni-NTA agarose columns and eluted in buffer containing 200 mM imidazol, 300 mM NaCl, 50 mM $Na_xH_yPO_4$, pH 8.0. Subsequently, samples were treated with 5 mM DTT for 30 min at 4 °C to fully reduce the protein. Remaining imidazole and DTT were removed using HiTrap desalting columns that were equilibrated with buffer containing 100 mM $Na_xH_yPO_4$, 0.1 mM DTPA, pH 7.4. Protein elution was monitored at 280 nm using an Äkta FPLC system. The protein concentration was determined spectrophotometrically using the molar extinction coefficient $\varepsilon_{280\ nm}$ = 21.43 mM^{-1} cm^{-1} as calculated for the primary sequence of the protein using the ProtParam ExPASy tool (http://web.expasy.org/protparam/). The thiol content of the proteins was analyzed with 5,5′-dithiobis-(2-nitrobenzoic acid) [17] revealing that 97% of the protein thiols were in a reduced state (data not shown).

2.5. Stopped-flow peroxidase measurements of recombinant wild type and mutant PfAOP

The oxidative half-reaction of 1 µM wild type *Pf*AOP, *Pf*AOP^{C117S}, *Pf*AOP^{C143S}, and *Pf*AOP^{L109M} with different hydroperoxides and peroxynitrite was analyzed using a SX-20 stopped-flow spectrofluorometer (Applied Photophysics). All activities were determined at 25 °C in 100 mM $Na_xH_yPO_4$ buffer containing 0.1 mM DTPA, pH 7.4. *Pf*AOP^{C117S} showed no change in fluorescence and served as a negative control. Two alternative methods were employed: