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Heme Regulatory Motifs in Heme Oxygenase-2 Form a Thiol/Disulfide Redox Switch That Responds to the Cellular Redox State*[S]

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Heme oxygenase (HO) catalyzes the rate-limiting step in heme catabolism to generate CO, biliverdin, and free iron. Two isoforms of HO have been identified in mammals: inducible HO-1 and constitutively expressed HO-2. HO-1 and HO-2 share similar physical and kinetic properties but have different physiological roles and tissue distributions. Unlike HO-1, which lacks cysteine residues, HO-2 contains three Cys-Pro signatures, known as heme regulatory motifs (HRMs), which are known to control processes related to iron and oxidative metabolism in organisms from bacteria to humans. In HO-2, the C-terminal HRMs constitute a thiol/disulfide redox switch that regulates affinity of the enzyme for heme (Yi, L., and Ragsdale, S. W. (2007) *J. Biol. Chem.* 282, 20156–21067). Here, we demonstrate that the thiol/disulfide switch in human HO-2 is physiologically relevant. Its redox potential was measured to be −200 mV, which is near the ambient intracellular redox potential. We expressed HO-2 in bacterial and human cells and measured the redox state of the C-terminal HRMs in growing cells by thiol-trapping experiments using the isotope-coded affinity tag technique. Under normal growth conditions, the HRMs are 60–70% reduced, whereas oxidative stress conditions convert most (86–89%) of the HRMs to the disulfide state. Treatment with reductants converts the HRMs largely (81–87%) to the reduced dithiol state. Thus, the thiol/disulfide switch in HO-2 responds to cellular oxidative stress and reductive conditions, representing a paradigm for how HRMs can integrate heme homeostasis with CO signaling and redox regulation of cellular metabolism.

Heme oxygenase (HO[3]; EC 1.14.99.3) catalyzes the O_2- and NADPH-dependent conversion of heme to biliverdin, carbon monoxide (CO), and iron in a reaction that is coupled to cytochrome P450 reductase. Then, biliverdin reductase catalyzes the NADPH-dependent reduction of biliverdin to the antioxidant bilirubin. Several recent reviews on HO (1–5) and biliverdin reductase (6) are available. HO is present in organisms from bacteria to eukaryotes and, as the only known enzyme that can degrade heme, plays a critical role in heme and iron homeostasis.

There are two major HO isoforms in mammals: inducible HO-1, which is ancient and widely distributed among organisms from bacteria to man, and constitutively expressed HO-2, which emerged 250 million years ago with the amniotes (7). HO-1 is found in most tissues and is highly expressed in spleen and liver (8). Conversely, HO-2 has a narrow tissue distribution, exhibiting high expression levels in the brain, testes, and carotid body (8, 9). Both HO-1 and HO-2 catalyze the NADPH- and cytochrome P450 reductase-dependent degradation of heme to CO, iron, and biliverdin, which is quickly reduced to bilirubin in the presence of biliverdin reductase (10). Controlling cellular heme concentrations is crucial because heme is required as a prosthetic group by regulatory and redox proteins, yet concentrations higher than 1 μM free heme are toxic (11). Thus, as the only mammalian proteins known to degrade heme, HOs play a key role in cellular heme homeostasis; furthermore, *in vitro* and *in vivo* studies of cellular and tissue injuries, such as oxidative stress and hemin-induced cytotoxicity, indicate that HO is cytoprotective (9).

HO-1 and HO-2 share high sequence and three-dimensional structural homology in their core domains (12, 13); however, their sequences diverge near their C termini, in which HO-2 contains two conserved heme regulatory motifs (HRMs), involving Cys^{265} in HRM1 and Cys^{282} in HRM2[4] (12, 14) (Fig. 1). It was shown recently that the HRMs in HO-2 do not bind heme *per se* but instead form a reversible thiol/disulfide redox switch that indirectly regulates the affinity of HO-2 for heme (14). However, for this redox switch to have any physiological consequence, the midpoint redox potential of the thiol/disulfide couple must be near the ambient intracellular redox potential, estimated to range from −170 to −250 mV (15).

The HRM has been proposed to constitute a heme-binding site (16, 17) that regulates key metabolic processes from bacteria to humans. The HRM consists of a conserved Cys-Pro core sequence that is usually flanked at the N terminus by basic amino acids and at the C terminus by a hydrophobic residue.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2 and Table 1.

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[3] The abbreviations used are: HO, heme oxygenase; HRM, heme regulatory motif; ICAT, isotope-coded affinity tag; MOPS, 4-morpholinepropanesulfonic acid; NAC, *N*-acetylcysteine; *t*-BuOOH, *tert*-butyl hydroperoxide; TCEP, tris(2-carboxyethyl)phosphine.

[4] HO-2 also contains another HRM involving Cys^{127} that does not appear to be involved in thiol/disulfide redox chemistry or enzymatic activity (14).

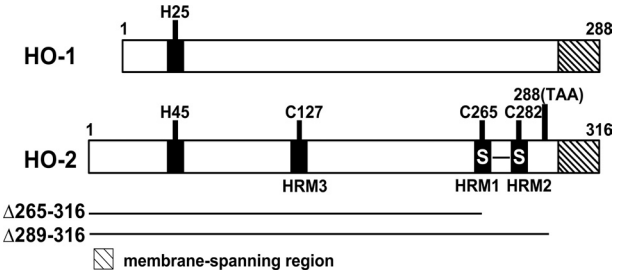

FIGURE 1. **Major structural regions in HO-1 and HO-2.** His^{25} in HO-1 or His^{45} in HO-2 is the heme-binding ligand.

HRM/heme interactions have been proposed to regulate the activity and/or stability of proteins that play central roles in respiration and oxidative damage (18, 19), coordination of protein synthesis and heme availability in reticulocytes (20, 21), and controlling iron and heme homeostasis (22–26). An important component of the last process is HO-2.

Here, we demonstrate that the C-terminal HRMs, which form a thiol/disulfide redox switch between Cys^{265} and Cys^{282}, exhibit a redox potential that falls well within the ambient cellular redox potential. By expressing HO-2 in bacterial and human cells and trapping the thiols using the isotope-coded affinity tag (ICAT) technique, it was shown that the redox state of the C-terminal HRMs in growing cells responds to the cellular redox state. The disulfide state is favored under oxidative conditions, and the dithiol state is predominant under reducing conditions. Thus, the HRMs act as a molecular rheostat that responds to the ambient intracellular redox potential and, based on earlier studies (14), controls activity of HO-2 by regulating heme binding to the enzyme.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of Human HO-2—The *in vitro* studies were performed using a truncated form of HO-2 (HO-2Δ289–316, denoted $HO\text{-}2_t$ here) that lacks the C-terminal membrane-binding region (14), whereas the full-length protein was used for experiments with growing cells. The C127A and F253W variants were generated from $HO\text{-}2_t$ using the QuikChange site-directed mutagenesis protocol (Stratagene, La Jolla, CA). HO-2 purification and enzymatic assay were performed as described previously (14).

HO-2 Stably Transfected HEK293 Cells—Full-length human HO-2 cDNA was subcloned into a pcDNA3.1(−) vector using XhaI and HindIII restriction sites. HEK293 cells were transfected with the recombinant pcDNA plasmid using TransIT-LT1 (Mirus, Madison, WI), as described by the manufacturer. Cells were cultured at 37 °C with selective medium containing 600 $\mu g \cdot ml^{-1}$ Geneticin every 3–4 days until Geneticin-resistant foci were identified. Then, colonies were picked and expanded in 60-mm^2 plastic dishes. The HO-2 expression levels in colonies were determined using Western blotting with an antibody generated against human HO-2.

Determination of the Midpoint Reduction Potential of the Thiol/Disulfide Redox Couple in HO-2—Two truncated forms of HO-2, $HO\text{-}2_t$, which contains all three HRMs, and C127A, which contains HRM1 and HRM2 but lacks HRM3, were used in these experiments because the full-length purified protein is subject to proteolysis and to precipitation. In a total 20-μl reaction system, 40 μg of $HO\text{-}2_t$ in Buffer A (50 mM KCl, 50 mM Tris-HCl, pH 7.5) was incubated with 2 μl of Buffer A and 8 μl of a solution containing various ratios of reduced (GSH) and oxidized (GSSG) glutathione, with the total GSH and GSSG concentrations fixed at 20 mM. The GSH/GSSG ratio was varied to establish a gradient of ambient redox potentials between −130 and −250 mV, calculated by the Nernst equation according to a midpoint reduction potential of −240 mV (27). The reaction mixtures were incubated at 37 °C with shaking for 2 h, and the reaction was terminated by adding 20 μl of 40% ice-chilled trichloroacetic acid and incubating on ice for 30 min. The suspension was centrifuged at 20,300 × *g* for 15 min at 4 °C. After removing the supernatant, the protein pellet was washed three times with −20 °C cold 100% acetone. The acetone-washed pellet was then resuspended in 20 μl of reaction buffer, containing 50 mM Tris-HCl, 4% SDS, and 20 mM purified Mal-PEG 5000 (Laysan Bio, Arab, AL), pH 5.0. After shaking at 25 °C for 30 min, 20 μl of nonreducing SDS loading buffer was added to the reaction buffer, and the samples were boiled for 5 min and analyzed by SDS-PAGE. After staining the gel with Coomassie Blue, the intensities of the bands from the unmodified and Mal-PEG 5000-modified HO-2 were measured using UN-SCAN-IT Gel 6.1 (Silk Scientific, Orem, UT), and the midpoint reduction potential of the thiol/disulfide couple from the C-terminal HRMs in HO-2 was calculated using the Nernst Equation 1. In this equation, E is the ambient potential in solution; E° is the midpoint reduction potential of HO-2; R is the universal gas constant: $R = 8.31\ J\ K^{-1}\ mol^{-1}$; T is the absolute temperature; F is the Faraday constant: $F = 9.65 \times 10^4\ C\ mol^{-1}$; and z is the number of electrons transferred in the reaction.

$$E = E^{\circ} - \frac{RT}{zF} \ln \frac{(\text{reduced HO-2})}{(\text{oxidized HO-2})} \qquad \text{(Eq. 1)}$$

Determination of the Redox States of the HRMs in HO-2 Using the ICAT Technique—OxICAT, a novel mass spectrometric method that combines thiol trapping with the ICAT technique to quantify oxidative thiol modifications, was performed as described previously (28) with minor modifications. For trapping the redox states of purified HO-2, 100 μg of as-isolated $HO\text{-}2_t$ or $HO\text{-}2_t$ that had been reduced with 1 mM tris(2-carboxyethyl)phosphine (TCEP) for 30 min was applied to a two-step alkylation procedure with light/heavy ICAT reagents as described previously. The alkylated protein was then digested with trypsin, and the cysteine-containing peptides, which are linked to biotin, were enriched on a cation-exchange cartridge followed by an avidin affinity cartridge (Applied Biosystems, Foster City, CA). After removing the biotin tag conjugated to the cysteine-containing peptides, samples were analyzed by nano-liquid chromatography/tandem mass spectrometry at the Michigan Proteome Consortium to quantify the amounts of reduced (dithiol) protein containing the light ICAT and oxidized (disulfide) protein containing the heavy ICAT adduct. For determining the redox state of the C-terminal HRMs, two peptides (Peptide A containing amino acid residues 264–285 and Peptide B containing residues 265–285), which differ in relative abundance (because both residues 264 and 265 are Lys)

among various experiments, were analyzed. As expected, the high-resolution mass spectra consist of envelopes of multiple peaks reflecting the presence of 0.018% ^{2}H, 1.11% ^{13}C, 0.45% ^{15}N, and 0.20% ^{18}O in each peptide. The m/z ratios for the oxidized form of these peptides (2877.0 for Peptide A and 2748.8 for Peptide B) are 18 mass units larger than those for the reduced protein (2858.9 and 2730.8, respectively).

For analysis of the redox state of HO-2 expressed in *Escherichia coli*, a pGEX4T-2 plasmid containing the full-length human HO-2 cDNA was transformed into *E. coli* strain BL21(DE3). Cells were grown in 50 ml of MOPS minimal medium containing 100 $\mu g \cdot ml^{-1}$ ampicillin at 37 °C. When the A_{600} reached 0.2, 0.01 mM isopropyl 1-thio-β-D-galactopyranoside was added to induce HO-2 expression. To generate oxidative stress, cells were treated with 400 μM *tert*-butyl hydroperoxide (*t*-BuOOH) or 1 mM diamide; and to generate a reducing environment, cells were treated with 1 mM *N*-acetylcysteine (NAC). In addition, the redox states of the HRMs were investigated under normoxic (continuously sparged with air) and anaerobic conditions. Anaerobic conditions were achieved by inoculating the aerobically growing cells into anaerobic MOPS minimal medium at a ratio of 1:500 (cells/medium). Cells were cultured at 37 °C during each of these treatments.

When the A_{600} reached 0.4, 0.2 ml of ice-cold 100% trichloroacetic acid was mixed with 1.8 ml of cell culture to lyse the cells, precipitate the proteins, and trap the thiol status. After washing with 5% trichloroacetic acid followed by 100% acetone (kept at −20 °C), the protein pellet was then applied to the two-step alkylation procedure with light/heavy ICAT reagents as described above.

The redox states of the C-terminal HRMs in HEK293 cells were determined as described for the *E. coli* system, with modifications. HO-2-transfected HEK293 cells were cultured in a 60-mm^2 plastic dish with Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, 10 units/ml penicillin, 100 mg/ml streptomycin, and 300 μg/ml Geneticin. After obtaining 50–60% cell confluence, 50% medium was replaced with fresh medium followed by treatment with *t*-BuOOH (200 μM, 12 h) or NAC (1 mM, 2 h). Then, the cells were processed by quickly washing three times with ice-cold phosphate-buffered saline, pipetting off the dishes with 1.8 ml of phosphate-buffered saline, immediately treating with 0.2 ml of ice-chilled 100% trichloroacetic acid, and briefly sonicating with a Misonix sonicator 4000. All of these steps were carried out on ice. The following ICAT alkylation steps were performed as described above, except that 1% Nonidet P-40 was added into the denaturing alkylation solution to facilitate resuspension of the protein pellet.

Intrinsic Fluorescence Quenching and CD Analysis—Tryptophan fluorescence is not detectable in as-isolated HO-2; therefore, we engineered a Phe to Trp substitution near the C-terminal HRMs. This F253W variant exhibits intrinsic tryptophan fluorescence that increases upon reduction of the disulfide bond, providing a straightforward and direct measure of the redox state of the thiol/disulfide couple. Intensity of the fluorescence emission at 340 nm of the F253W (200–500 nM) variant of HO-2 was measured with a Shimadzu RF-530 1 PC spectrofluorophotometer at room temperature. CD measurements were performed at 4 °C with a JASCO J-715 instrument. For secondary structure analysis, experiments were performed in a 0.1-mm path length cell with 20 μM HO-2_t in 10 mM potassium phosphate buffer, pH 7.5. The F253W variant exhibited ~50% of the specific activity of the wild-type protein.

RESULTS AND DISCUSSION

Determination of the Redox Potential of the Thiol/Disulfide Redox Switch in HO-2—To test the hypothesis that the C-terminal HRMs in HO-2 form a physiologically relevant redox switch that is poised to sense the intracellular redox potential, the midpoint reduction potential of the thiol/disulfide redox couple was determined. HO-2_t[5] samples were equilibrated with solutions of oxidized/reduced glutathione (GSSG/GSH) at ratios that established a gradient of ambient redox potentials between −130 and −250 mV. Then, the proteins were precipitated by treating the samples with trichloroacetic acid and alkylated with Mal-PEG 5000, which traps the free thiols as an adduct, resulting in a mass increase of 5 kDa/thiol modification. Thus, the oxidized (nonmodified disulfide, *lower band*) and reduced (modified, *upper band*) forms of HO-2_t can be quantified conveniently by analyzing the migration pattern on nonreducing SDS-PAGE (Fig. 2*A*).[6] To assign the titrated disulfide bond to Cys^{265} and Cys^{282} unambiguously, we used a form of HO-2 containing all three HRMs (HO-2_t) as well as the HO-2_t variant containing only the two C-terminal HRMs (C127A) (Fig. 2). After quantifying the ratio of oxidized/reduced protein at each ambient redox potential and fitting the data to the Nernst equation, the calculated midpoint reduction potentials of the thiol/disulfide redox switches in C127A (−191 mV) and HO-2_t (−199 mV) (Fig. 2*B*) were nearly identical.

To confirm the value of the midpoint potential determined by the Mal-PEG 5000 approach, we performed intrinsic tryptophan fluorescence experiments of an HO-2_t variant (F253W) in which a Phe to Trp substitution was engineered near the C-terminal HRMs. Fluorescence quenching of excited Trp by nearby disulfide bridges is common in proteins and is proposed to occur through an electron transfer mechanism from excited Trp to the disulfide bond (29). The activity of the F253W variant is ~2-fold lower than that of HO-2_t (supplemental Fig. 1*A*), indicating that major structural changes have not occurred by the replacement of Phe with Trp at this position. Reduction of the disulfide bond in the F253W variant leads to a 2-fold increase in fluorescence intensity (supplemental Fig. 1*B*). When solutions of the F253W variant are equilibrated at different redox potentials, set by establishing a gradient of GSSG/GSH ratios as described above, the fluorescence intensity is quenched by the disulfide bond. Fitting the data to the Nernst equation provides a midpoint reduction potential of −200 mV (Fig. 2*B*). Thus, both thiol modification and intrinsic trypto-

[5] Because purified full-length HO-2 is unstable and subject to precipitation, we performed redox studies with a truncated form (HO-2Δ289-316) of the protein, herein termed HO-2_t, which lacks the membrane-spanning region.

[6] We assign the middle band to represent an intermediate state with a single Mal-PEG 5000 adduct. This intermediate is likely to be the sulfenate form of Cys^{265} of HO-2, which is unreactive with Mal-PEG 5000 until it undergoes reduction with a thiol reactive reagent (glutathione in this case). OxICAT analyses, described below, are consistent with this assignment.

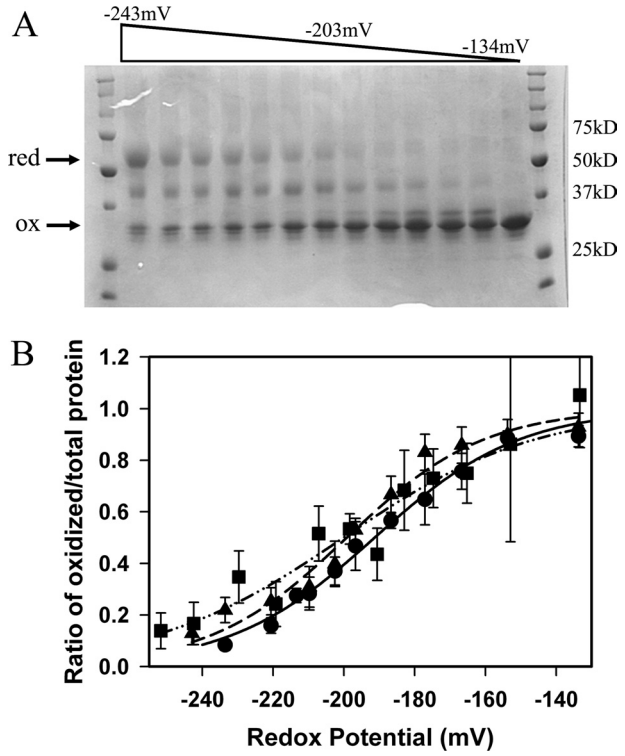

FIGURE 2. **Measurement of the midpoint redox potential of the thiol/disulfide couple in HO-2$_t$.** C127A was used in the alkylation experiment to determine the redox potential of the C-terminal HRMs. The ambient redox potential, set by variation of the GSSG/GSH ratio, ranged from −133 to −252 mV (see details under "Experimental Procedures"). *A*, SDS-PAGE analysis of the alkylation of C127A by Mal-PEG 5000. *red*, reduced; *ox*, oxidized. *B*, Nernst analysis of results from nonreducing PAGE shown in *A* and fluorescence quenching studies of F253W. The redox potential of the C-terminal HRMs, analyzed using the Nernst equation, provided $E^o = -191 \pm 1$ mV for C127A (*filled circles*), $E^o = -199 \pm 2$ for HO-2$_t$ (*filled triangles*), and $E^o = -200 \pm 3$ mV for F253W (*filled squares*).

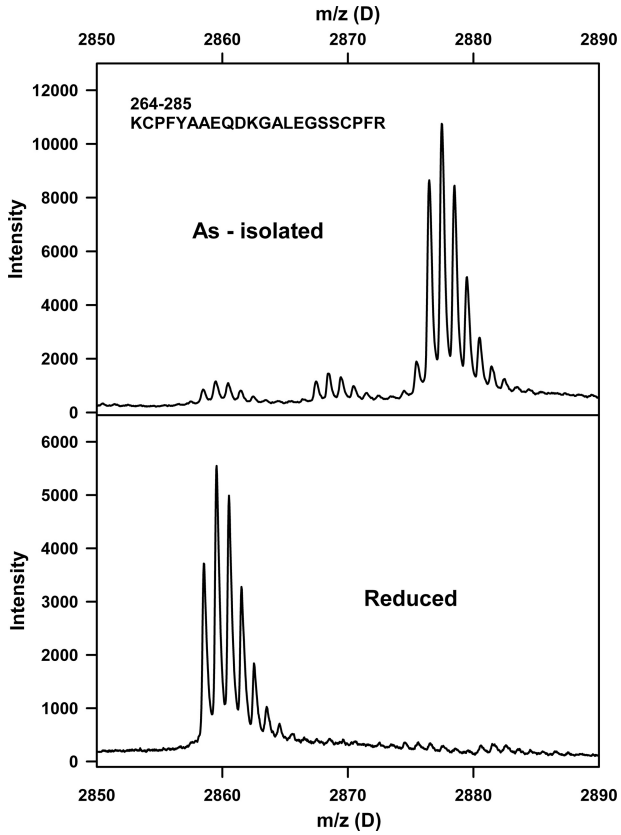

FIGURE 3. **OxICAT analysis of the redox state of the C-terminal HRMs in purified HO-2.** Purified HO-2$_t$ was treated as described under "Experimental Procedures." *Top panel*, as-isolated HO-2$_t$. *Bottom panel*, TCEP-reduced HO-2$_t$, generated by incubating 100 μg HO-2$_t$ with 1 mM TCEP for 30 min.

phan fluorescence experiments provide similar values of −190 to −200 mV for the midpoint potential of the thiol/disulfide redox switch in HO-2$_t$.[7] This value is within the range of redox potentials for proteins known to catalyze key thiol/disulfide oxidoreductase reactions, from −122 mV for the oxidase DsbA (30) to −270 mV for the reductase thioredoxin (31). It lies precisely within the range measured for the intracellular redox potential, from −170 (15) to −325 mV (32), indicating that the thiol/disulfide redox switch in HO-2 is poised to respond to the redox state within the cell.

To investigate the structural differences between the oxidized and reduced forms of HO-2, the CD spectra of as-isolated and TCEP-reduced HO-2 were recorded. These spectra are nearly identical (supplemental Fig. 1*C*), indicating that if there are any secondary structural changes in HO-2 upon oxidation or reduction of the thiol/disulfide redox switch, they would be minor local changes.

Response of the Thiol/Disulfide Redox Couple to Alteration in the Cellular Redox Potential—To test further the hypothesis that the thiol/disulfide switch in HO-2 responds to changes in intracellular redox conditions, the redox state of the HRMs was measured by OxICAT (28). ICAT consists of an iodoacetamide group linked to a cleavable biotin affinity tag via an isotopically light (^{12}C) or heavy (^{13}C) nine-carbon linker (33).

Before initiating experiments with growing cells, we performed the OxICAT analysis on pure HO-2. To determine the redox status of freshly purified protein, we treated HO-2$_t$ with the light ICAT reagent, reduced all existing oxidative thiol modifications, and alkylated all newly reduced thiols with heavy ICAT. Thus, a peptide containing a free Cys thiol(ate) will have an m/z value that is 9 units smaller than one containing a Cys that is engaged in a disulfide bond. Mass spectral analysis of the affinity-purified tryptic peptide harboring HRM1 and HRM2 (Peptide A, residues 264–285) generated from as-isolated HO-2 reveals peaks at 2858.9, 2868.0 (+9), and 2877.0 (+18), with the major peak at 2877.0, resulting from labeling with two heavy ICAT molecules (Fig. 3). Thus, the majority of the as-isolated protein contains cysteines in HRM1 and HRM2 that are engaged in an intramolecular disulfide bond. In contrast, when HO-2 is reduced by TCEP before the OxICAT procedure, the light ICAT-labeled peptide is in significantly greater abundance. Thus, the cysteines of HRM1 and HRM2 in purified HO-2 are mainly (90.6%) in the disulfide state, and TCEP converts the HRMs nearly quantitatively (94.2%) to the dithiol state (Fig. 3). This result indicates that HO-2 is highly oxidation-sensitive and accumulates in its disulfide bond form when reducing agents are absent during its purification.

The minor peak in Fig. 3 at m/z 2868.0 (+9) in the as-isolated protein is likely caused by the presence of a small amount of the

[7] These results also demonstrate nicely that the trapping experiments with Mal-PEG 5000 are fast enough to freeze the thiol redox state.

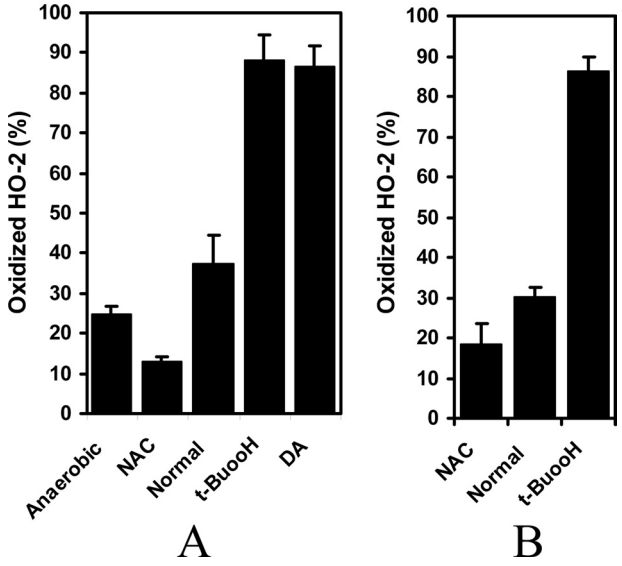

FIGURE 4. **Redox state of the C-terminal HRMs of HO-2 expressed in *E. coli* and in HEK293 cells under different cellular redox conditions.** The OxICAT technique was applied to investigate the redox states of the C-terminal HRMs of HO-2 expressed under different conditions, as described under "Experimental Procedures." *A*, wild-type HO-2 was expressed in *E. coli* strain BL21(DE3) and grown at 37 °C under normoxic or anaerobic growth conditions or treated with 1 mM NAC (2 h), 400 μM *t*-BuOOH (15 h), or 1 mM diamide (*DA*; 10 min), as described under "Experimental Procedures." *B*, wild-type HO-2 was expressed in HEK293 cells and grown at 37 °C under normoxic conditions or treated with 1 mM NAC (2 h) or 200 μM *t*-BuOOH (12–15 h), as described under "Experimental Procedures."

sulfenate (S-OH) of Cys^{265} in the sample. The peak has a +9 value of *m*/*z* because the S-OH does not react with iodoacetamide; however, TCEP reduces the S-OH to the SH in the second step of the OxICAT protocol. Furthermore, the intermediate band shown in Fig. 2*A* is probably caused by a small amount of Cys-sulfenate in the sample (see above). Similarly, a sulfenate intermediate was reported in OxICAT analysis of Hsp33 and GapA (28). Our assignment of Cys^{265}-S-OH is consistent with earlier studies involving the C127A/C282A HO-2_t variant, in which the sulfenate of Cys^{265} was trapped as an adduct with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole and was identified as the sulfonate by mass spectrometry; thus, we proposed that the sulfenate of Cys^{265} is an intermediate in the formation of the disulfide (14).

To determine whether the thiol/disulfide switch in HO-2 responds to changes in intracellular redox conditions, full-length HO-2 was expressed in human and *E. coli* cells, and the redox states of the HRMs were determined by the OxICAT method (Fig. 4*A*) (also see supplemental Fig. 2 and supplemental Table 1). When *E. coli* was grown under aerobic conditions, the C-terminal HRMs were distributed nearly equally between the reduced (63%) and oxidized (37%) states, which corresponds well with the determined redox potential of HO-2_t, suggesting that truncation of the membrane-spanning region has little effect on the thiol/disulfide redox potential and that HO-2 is in equilibrium with the cellular GSSG/GSH ratio. The proportion of reduced HRMs increased to 75.0% or 87.0% when cells were grown either anaerobically or treated with 1 mM NAC, respectively. When cells were subjected to oxidative stress generated by the addition of 1 mM diamide or 400 μM *t*-BuOOH, however, the proportion of reduced HRMs decreased to 11.0% and 12.2%, respectively.

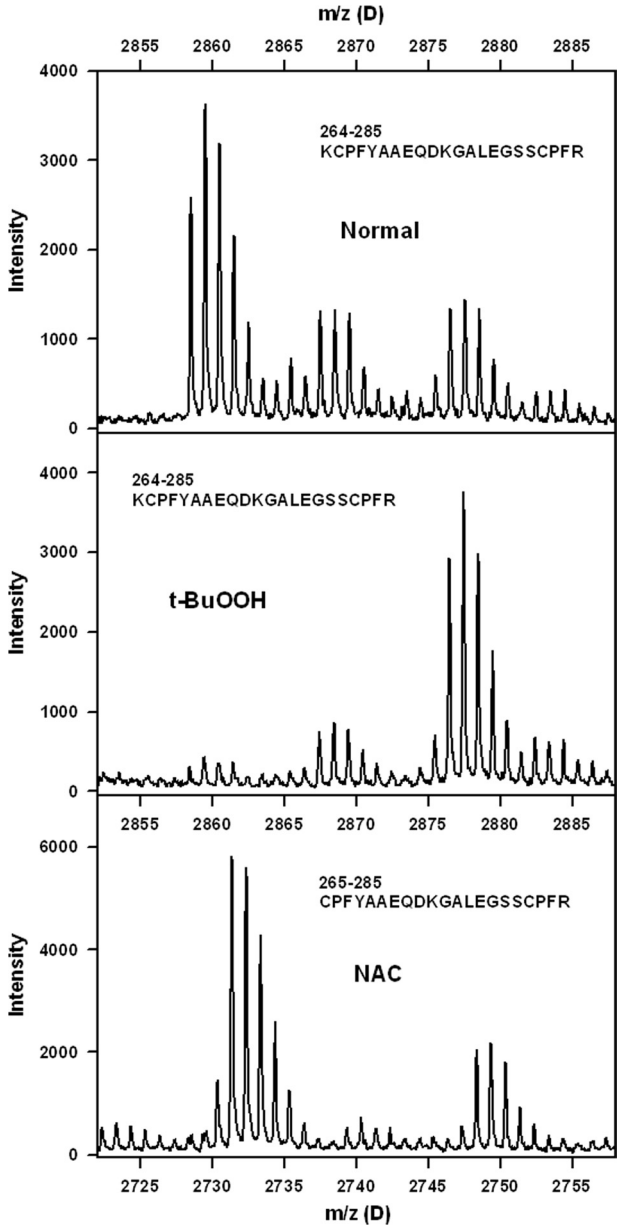

FIGURE 5. **Representative mass spectrometric results of the redox states of C-terminal HRMs in HO-2 expressed in HEK293 cells grown under different conditions.** Full-length HO-2 was stably expressed in a HEK293 cell line as described under "Experimental Procedures." The OxICAT technique was used to investigate the redox states of C-terminal HRMs when cells were treated with 1 mM NAC for 2 h (*top panel*), normal growth conditions (*middle panel*), or 200 μM *t*-BuOOH for 12–15 h (*bottom panel*).

Similar experiments were performed with stably transfected HEK293 cells containing the full-length HO-2 gene, which was integrated into the genome and constitutively expressed (Fig. 5). When this cell line was cultured under normoxic conditions, the C-terminal HRMs were mostly (70%) in the reduced dithiol state, whereas under oxidative stress conditions (200 μM *t*-BuOOH), the HRMs were converted predominantly (86%) to the disulfide state; in contrast, after treatment with 2 mM NAC, the HRMs were found mostly (82%) in the reduced dithiol state (Figs. 4*B* and 5) (see also supplemental Table 1). Thus, the C-terminal HRMs in HO-2 form a physiologically relevant redox rheostat that responds to the intracellular redox poten-

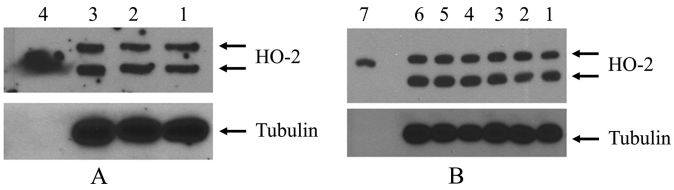

FIGURE 6. **Stable expression of HO-2 in HEK293 cells.** *A*, cells were cultured under different redox conditions. *Lane 1*, 1 mM NAC (2 h); *lane 2*, normal conditions; *lane 3*, 200 μM *t*-BuOOH (12 h); *lane 4*, purified HO-2_t. *B*, cells were cultured with different hemin additions. *Lane 1*, normal conditions; *lane 2*, 100 nM hemin (20 h); *lane 3*, 1 μM hemin (20 h); *lane 4*, 10 μM hemin (20 h); *lane 5*, 50 μM hemin (20 h); *lane 6*, 100 μM hemin (20 h); *lane 7*, purified HO-2_t.

tial. The amount of HO-2 that accumulates in the stably transfected HEK293 cell line is unaffected by treatment with oxidants or reductants or by adding hemin to the cells (Fig. 6). This is consistent with prior studies, which demonstrated that expression of HO-1, but not HO-2, is induced by oxidative stress (10). Thus, the shifts in intracellular redox potential alter the redox states of the C-terminal HRMs in HO-2 but do not appear to alter the expression or stability of HO-2.

Conclusions—HO-2 appears to interlink redox and heme homeostasis; the midpoint reduction potential of the thiol/disulfide redox switch is poised near the ambient intracellular redox potential, and the K_d for heme (0.03 μM) is near the free cellular heme pool (0.03–1 μM) (34). We found that HO-2 expression levels are unaffected by alteration in the cellular redox state (or growth in excess heme); however, the thiol/disulfide switch does exhibit a robust response to redox conditions. When cells are exposed to oxidative conditions, the Cys residues in the C-terminal HRMs in HO-2 are in the disulfide state, which favors heme binding; however, under reducing conditions, the lower affinity dithiol state predominates. Thus, these HRMs of HO-2 act as a redox rheostat, which regulates the binding of heme and, therefore, HO-2 activity as a Nernstian function of the redox state of the cell. Under oxidative stress conditions, HO-2 can degrade heme rapidly, increasing the levels of CO and biliverdin; however, under hypoxic (or otherwise reduced) conditions, where the HRMs are in the dithiol state, the K_d (0.35 μM) rises well above the free heme pool (14). This redox switching mechanism could potentially connect the many physiological processes regulated by heme homeostasis and CO signaling to the cellular redox state. Such processes include oxygen sensing by the carotid body (the oxygen chemosensor in mammals), regulation of T cell function, blockade of oxidant formation in neurons where HO-2 appears to be the sole source of CO biogenesis (35), and control of heme homeostasis (9, 36, 37). Our results also suggest that the myriad proposed functions ascribed to HRM/heme interactions in various proteins might actually be mediated by thiol/disulfide redox reactions. Thus, HO-2 may represent a paradigm for how HRMs can integrate heme, CO, and redox regulation of metabolism.

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