Supporting Information

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SI Text

Calculations.

Calculating the fraction of diamide-accessible PSH equivalents in HEK cells. Data are obtained from Table S1.

Diamide-accessible PSH = $((22.3 - 12.7) \times 10^{-3}$ SH/aa)/$(22.3 \times 10^{-3}$ SH/aa) = 0.43.

Calculations Performed to Obtain Fig. 5.

Distribution of total SH/aa in thiols and disulfides (Fig. 5A). The described calculations are performed with data from HEK cells not exposed to diamide as shown in Tables S1 and S2.

Total SH/aa found as thiols = GSH + PSH = $(22.3 + 8.7) \times 10^{-3} = 31 \times 10^{-3}$.

Total SH/aa found as disulfides = PSSP + PSSG + GSSG = $(1.39 + 0.0088 + 0.81) \times 10^{-3} = 2.21 \times 10^{-3}$.

Total SH/aa = Total thiols + total disulfides = $(31 + 2.21) \times 10^{-3} = 33.21 \times 10^{-3}$.

% SH/aa equivalents found as thiols = $(31/33.21) \times 100 = 93\%$.

% SH/aa equivalents found as disulfides = $(2.21/33.21) \times 100 = 7\%$.

Distribution of diamide oxidation equivalents on GSH and PSH (Fig. 5B). The calculations are performed with data from HEK cells shown in Tables S1 and S2. The difference in the PSH pool (ΔPSH) and GSH pool (ΔGSH) before and after diamide treatment was used to calculate the relative distribution of diamide equivalents.

PSH equivalents consumed by diamide = ΔPSH = $PSH_{no\ treatment} - PSH_{diamide} = (22.3 - 12.7) \times 10^{-3}$ SH/aa = 9.6×10^{-3} SH/aa.

GSH equivalents consumed by diamide = ΔGSH = $GSH_{no\ treatment} - GSH_{diamide} = (8.7 - 1.2) \times 10^{-3}$ SH/aa = 7.5×10^{-3} SH/aa.

Total SH/aa equivalents consumed by diamide = ΔPSH + ΔGSH = $(9.6 + 7.5) \times 10^{-3}$ SH/aa = 17.1×10^{-3} SH/aa.

% SH/aa of diamide consumed by PSH = [ΔPSH/(ΔPSH + ΔGSH)] × 100 = $[(9.6 \times 10^{-3}$ SH/aa)/$(17.1 \times 10^{-3}$ SH/aa)] × 100 = 56%.

% SH/aa of diamide consumed by GSH = [ΔGSH/(ΔPSH + ΔGSH)] × 100 = $[(7.5 \times 10^{-3}$ SH/aa)/$(17.1 \times 10^{-3}$ SH/aa)] × 100 = 44%.

Detailed Experimental Procedures. ***Cell culture, diamide exposure, and sample preparation.*** HEK and HeLa cells were maintained in α-MEM (Invitrogen), supplemented with 10% (vol/vol) FCS (HyClone) at 37 °C under 5% CO_2. Cells were seeded in a 10-cm dish, so they could reach 95% confluency the following day. Cells were trypsinized, resuspended in medium without FCS, and harvested by centrifugation. To eliminate traces of trypsin, cells were washed with 10 mL of PBS. After centrifugation, cells were resuspended in 900 μL of PBS with or without 5 mM diamide (Fluka) and incubated for 5 min at 37 °C with shaking (500 rpm, Eppendorf thermoshaker). Cells were lysed by adding 100 μL of ice-cold 100% (wt/vol) TCA and stored on ice for 30 min, followed by centrifugation at 20,000 × *g* for 10 min at 4 °C. The TCA supernatant was used for quantification of low-molecular-mass thiols. If quantification was not performed immediately, the TCA supernatant was frozen and kept at −80 °C until later use. The TCA pellet was washed in 10% TCA by 4 rounds of sonication and centrifugation.

Quantification of glutathione. Soluble GSH and GSSG levels in the TCA supernatant were determined by HPLC using derivatization with NPM (Fluka) essentially as described in ref. 1 but optimized for TCA supernatants. Briefly, for determination of GSSG free thiols were blocked by adding 5 μL of 10 mM NEM (Sigma-Aldrich) to 20 μL of TCA supernatant. The pH was increased to 8 by adding a mixture containing 50 μL of 0.1 M Tris·HCl, pH 8.0, 118 μL of water, and 12 μL of 1 M NaOH. Excess NEM was quenched with 2-mercaptoethanol followed by reduction and derivatization with TCEP (Sigma–Aldrich) and NPM as described. For quantification of total GS, 20 μL of the supernatant (appropriately diluted) was combined with 50 μL of 0.1 M Tris·HCl, pH 8.0, and 170 μL of 0.7 M NaOH followed by reduction and thiol derivatization. The reliability of the method was ascertained by the full recovery of exogenously added GSSG (Sigma-Aldrich) to cell extracts implying a quantitative reduction of disulfides with TCEP. As a control for the alkylating efficiency, GSH (Sigma-Aldrich) was added to cell extracts before addition of NEM. A recovery of 1.75% was obtained corresponding to the GSSG contamination in the commercial GSH stock.

Quantification of PSH. P1 was solubilized by sonicating in 500 μL of 0.4 M sodium citrate, 1 mM EDTA, 5% SDS, pH 4.5. For quantification of PSH, 50 μL of the sample was mixed with 300 μL of 0.4 M citrate, 1 mM EDTA, 0.3% SDS, pH 4.5, and 3 μL of 55 mM 4-DPS (Sigma-Aldrich), dissolved in ethanol, and incubated for 30 min at room temperature. To avoid SDS precipitation, 650 μL of 0.1 M sodium citrate, 4 M urea, pH 4.5, was added. The 4-TP reaction product was quantified using HPLC with absorbance detection at 324 nm, as described in ref. 2. The concentration of 4-TP was determined by relating the integrated peak area to a standard curve made with known amounts of cysteine.

Quantification of PS_{ox} and total PS. Free protein thiols were blocked by sonicating P2 in 500 μL of 0.5 M Tris·HCl, 1 mM EDTA, 5% SDS, 20 mM NEM, pH 8.3. The alkylation was allowed to proceed for 15 min at 50 °C. 50 μL of the sample was mixed with 90 μL of 0.5 M Tris·HCl, 1 mM EDTA, 1% SDS, pH 8.3., 10 μL of water, 10 μL of hexanol, and 20 μL of freshly prepared 30% (wt/vol) alkaline BH (Sigma-Aldrich). Reduction, followed by thiol quantification, was performed as described in ref. 2. Total PS was experimentally measured by sonicating the P3 in buffer without NEM.

Quantifying PSSG. P4 was dissolved by sonication in 500 μL of 200 mM Bicine, 8 mM EDTA, 6 M urea, pH 9.2 (8.5 at 60 °C). 90 μL of the dissolved sample was mixed with 5 μL of 42 mM THP (Calbiochem) and 5 μL of 3% (wt/vol) SBD-F (Fluka). After 1 h of incubation at 60 °C the samples were quenched by addition of HCl to a final concentration of 0.17 M. Before HPLC analysis, samples were centrifuged for 10 min at 16,000 × *g* to eliminate any cell precipitates. Samples were kept in the dark for 4 °C until analysis by HPLC as described below. Under these conditions the SBD-labeled thiols are stable for 2 weeks (3). Quantification of SBD-labeled thiols was performed by HPLC using a Phenomenex Luna C18 (2) column (5μ 100 Å, 150 × 4.6 mm) protected by a C18 4 × 3 mm guard column. A 90-μL aliquot was injected onto the column and eluted isocractically in 75 mM sodium citrate, pH 2.9, 2% methanol at a flow rate of 0.8 mL/min. The SBD-derivatized thiols were detected by fluorescence with excitation and emission at 386 nm and 516 nm, respectively. Thiol concentrations were determined by relating the integrated peak areas to a standard curve based on known amounts of GSH.

Identification of low-molecular-weight thiols in TCA supernatant. Twenty microliters of the TCA supernatant was combined with 40 μL of 200 mM Bicine, 8 mM EDTA, pH 9.2, 15 μL of water, and 12

μL of 1 M NaOH to raise the pH to 9. Disulfides were reduced by THP, derivatized by SBD-F, and analyzed by HPLC as described for the PSSG quantification.

Total protein quantification. The amount of proteins in the TCA pellets was determined by quantifying amino acids after acid hydrolysis using a modification of the method described by Starcher (4). Proteins in the dissolved TCA pellets were hydrolyzed by adding HCl to a final concentration of 6 M followed by incubation at 100 °C overnight (at least 16 h). The acid hydrolysate was evaporated to dryness and redissolved in 200 μL of 50 mM sodium phosphate, pH 8.0. Twenty microliters of the sample was mixed with 480 μL of water and 500 μL of ninhydrin reagent. The sample was incubated for 10 min at 100 °C and placed on ice for 5 min, and the absorbance was measured at 570 nm. Amino acid concentrations were determined by relating the absorbance to a standard curve based on known amounts of tyrosine. To avoid interfering contributions from primary amines in the buffer used to solubilize TCA pellets, buffer samples was subjected to acid hydrolysis and ninhydrin analysis. The ninhydrin absorbance of the buffers used to solubilize P1–P3 (Fig. 1) was either below detection limit or very low (<5% of the total absorbance) and could be subtracted from the cell measurements. However, for the solubilization of P4, urea was used as denaturant, and the contribution to the ninhydrin color was significant. Consequently, P4 was divided into SQ and PQ before solubilization in buffers with or without urea, respectively. The reliability of the method was ascertained by the full recovery of exogenously added tyrosine to cell extracts. In addition, a known amount of lysozyme was subjected to hydrolysis and ninhydrin quantification and a recovery of 80% of the expected amino acid amount was obtained (data not shown).

Preparation of ninhydrin reagent. One hundred milligrams of ninhydrin (Merck) was dissolved in 7.3 mL of dimethyl sulfoxide (DMSO) and 2.5 mL of 1.65 M lithium acetate, pH 4.25. The color of the mixture was light yellow. Ninhydrin was reduced to the reactive hydrindantin by the addition of 200 μL of 0.66 M $SnCl_2$ (75 mg dissolved in 500 μL of DMSO). The reduction was allowed to proceed for 5 min, until the reagent had turned purple. The reagent was prepared freshly and used immediately for amino acid quantification.

Analytical techniques. The concentration of thiols used for standard curves was determined using 0.1 mM DNTB in 0.1 M potassium phosphate buffer at pH 7.0 using the extinction coefficient of 14,150 $M^{-1}{\cdot}cm^{-1}$ for 2-nitro-5-thiobenzoic acid (5). GSSG concentrations were quantified from the absorbance at 248 nm [ε = 382 $M^{-1}{\cdot}cm^{-1}$ (6)]. The concentration of lysozyme (Sigma-Aldrich) was determined from the absorbance at 280 nm (ε = 38,469 $M^{-1}{\cdot}cm^{-1}$) as described in ref. 7. The concentration of tyrosine was determined from the absorbance at 275 nm (ε = 1400 $M^{-1}{\cdot}cm^{-1}$). All concentrations were determined by 3 independent measurements.

Instrumentation. HPLC analysis was performed with a Hewlett-Packard 1100 series LC system coupled to a 1200 series autosampler (Agilent Technologies). In all experiments the autosampler temperature was set to 4 °C. Peak areas were integrated using the accompanying software (HP ChemStation).

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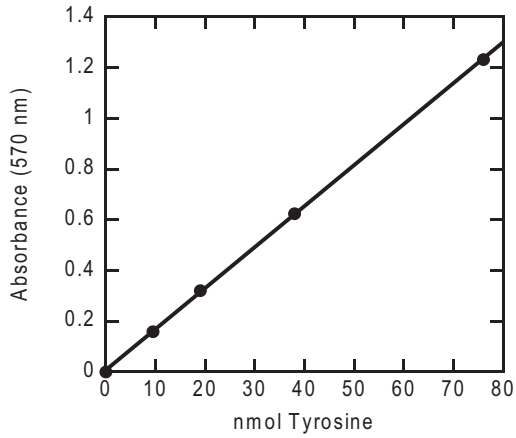

Fig. S1. Standard curve for amino acid quantification based on the reaction between tyrosine and ninhydrin. Different amino acids vary somewhat in their color yields in the ninhydrin reaction (1). We decided to use tyrosine as standard amino acid as the relative color yield compared with leucine (the most common amino acid in eukaryotic proteins (2)) is close to unity and due to tyrosines favorable spectroscopic properties, which conveniently can be used to determine the exact concentration in the standard solution. Various concentrations of tyrosine (19–152 μM) were incubated with ninhydrin reagent (prepared as described in *Materials and Methods*) for 10 min at 100 °C. After incubation on ice for 5 min the absorbance was measured at 570 nm.

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2. Pe'er I, *et al.* (2004) Proteomic signatures: Amino acid and oligopeptide compositions differentiate among phyla. *Proteins* 54:20–40.

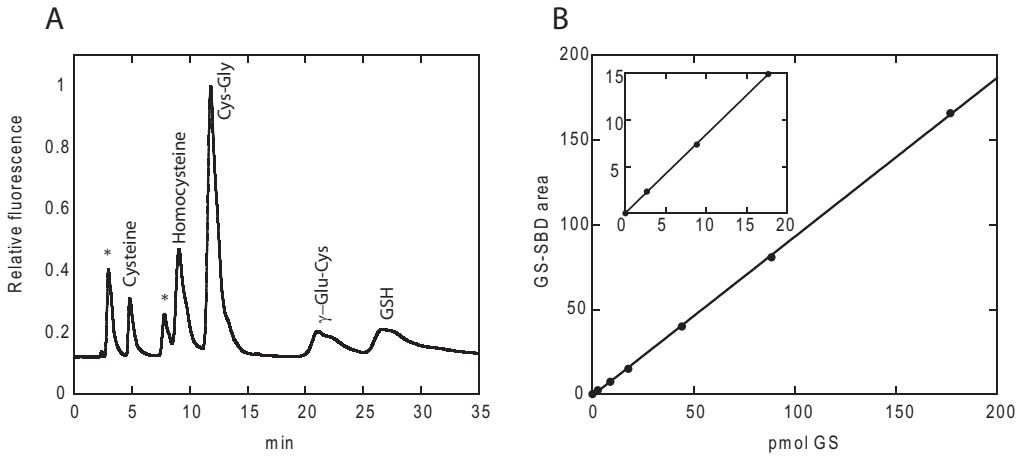

Fig. S2. HPLC assay for quantification of protein glutathionylation. Sulfhydryl compounds were reduced with 2.1 mM THP and derivatized with 6.4 mM SBD-F in the presence of 6 M urea. Samples were injected onto a Phenomenex Luna C18 column and eluted isocractically in 75 mM sodium citrate, pH 2.9, 2% methanol. (*A*) HPLC chromatograms of SBD-labeled thiol compounds. *, solvent peak. (*B*) Standard curve for glutathione quantification made with various concentrations of GSH. Linearity was observed in the range of 0–200 pmol with a detection limit of 2 pmol. GS quantified from cell pellets were typically in the range of 5–15 pmol. This range of the standard curve is shown in *Inset*.

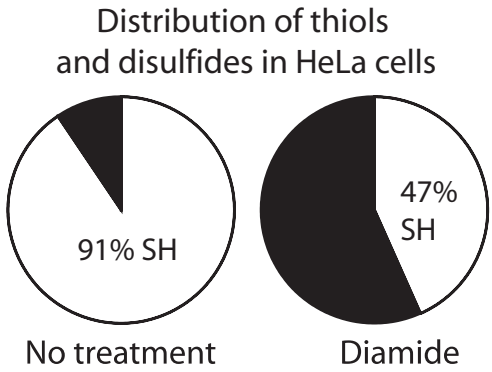

Fig. S3. Redistribution of total thiol and disulfide pools upon diamide exposure of HeLa cells. Cells were exposed to 5 mM diamide for 5 min before TCA quenching. Total thiols pools (GSH + PSH) are shown in white, and total disulfide pools (PSSP + GSSG + PSSG) are shown in black.

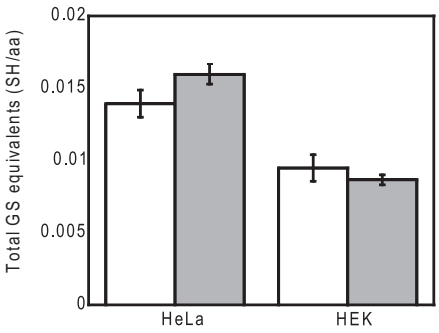

Fig. S4. Quantitative recovery of total GS equivalents after diamide. Total cellular GS equivalents were calculated by addition of SH/aa values of [total soluble GS] and [GS in PSSG]. White bars shows the values for cells grown under normal conditions and gray bars shows the values after cells have been exposed to 5 mM diamide for 5 min.

Table S1. Distribution of protein sulfhydryl equivalents in HEK and HeLa cells

	(SH/aa) $\times 10^{-3}$			
	HEK		HeLa	
Thiol or disulfide	No treatment	With diamide	No treatment	With diamide
PSH	22.3 ± 0.5	12.7 ± 0.8	25 ± 1	10.9 ± 0.5
PS in PSSP*	1.39 ± 0.07	5.0 ± 0.5	2.6 ± 0.1	4.8 ± 2
PS in PSSG	0.0088 ± 0.0005	3.2 ± 0.1	0.0142 ± 0.0009	3.6 ± 0.6

Values are given as the means ± SD.
*PS in PSSP was calculated by subtracting (2 × PS in PSSG) from PS_{ox}.

Table S2. Distribution of glutathione equivalents in HEK and HeLa cells

	(SH/aa) × 10^{-3}			
	HEK		HeLa	
Thiol or disulfide	No treatment	With diamide*	No treatment	With diamide*
GS in PSSG	0.0088 ± 0.0005	3.2 ± 0.1	0.0142 ± 0.0009	3.6 ± 0.6
GS in GSSG	0.81 ± 0.08	4.3 ± 1.0	1.26 ± 0.06	6.5 ± 1
GSH†	8.7 ± 1	1.2 ± 1.3	12.6 ± 1	5.9 ± 2

Values are given as the means ± SD.
*Quantified GSSG levels were corrected for postlysis diamide oxidation, as described in *Experimental Procedures*.
†GSH is calculated by subtracting [GS in GSSG] from [total soluble GS].

Table S3. Viability of HeLa cells after diamide oxidation

	No. of cells × 10^6	
Day	No treatment	With diamide
0	1.9	1.9
1	4.0	2.0
2	ND	3.6

Fifty percent of confluent HeLa cells in 10-cm dishes were exposed to 10 mL of 5 mM diamide in PBS for 5 min at 37 °C. Diamide was removed, the cells were washed twice with 10 mL of PBS, fresh medium was added. The cells were incubated for 1 or 2 days at 37 °C before they were counted. Dead cells were stained with trypan blue to ensure that only viable cells were counted. ND, not determined.