The proportion of Met80-sulfoxide dictates peroxidase activity of human cytochrome *c*

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SUPPLEMENTARY INFORMATION

Kinetics of heme bleaching by hydrogen peroxide

Kinetics of heme bleaching was followed at 410 nm for WT and all variants and for varying hydrogen peroxide concentrations. Disappearance of the Soret band was followed using reaction mixtures (100 µL) that contained cytochrome c (5 µM), H_2O_2 (25-300 µM) and sodium phosphate buffer (pH 5.4, 80 mM). Reactions were initiated by addition of hydrogen peroxide and spectra (300-600 nm) monitored on a 96 well plate using a MultiSkan GO microplate spectrophotometer (Thermo Scientific). Data were fitted using a single exponential using Prism software from Graphpad.

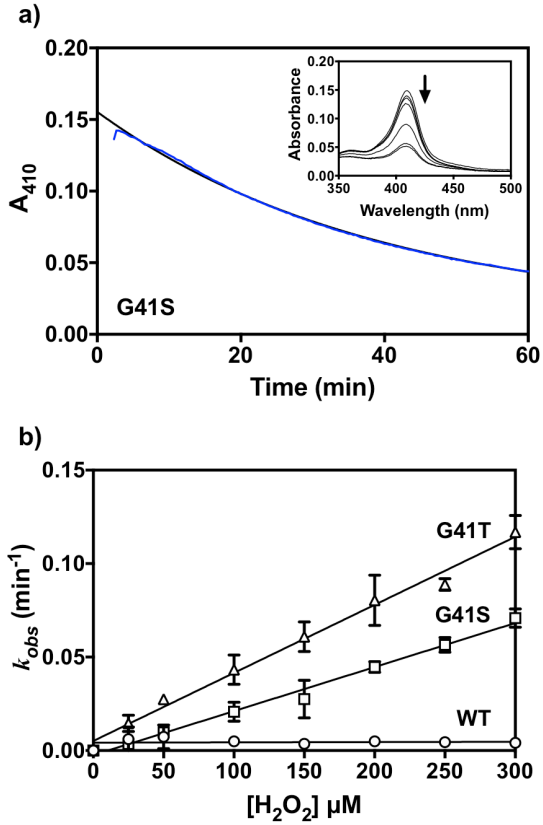

Figure S1. Impact of H_2O_2 on the Soret band of cytochrome c and variants. Cytochrome c (5 µM) was incubated in the presence of H_2O_2 (0-300 µM) in phosphate buffer (pH 5.4). Absorption spectra from 300-600 nm were monitored, and the absorbance at 410 nm plotted against time. a) Representative data, using G41S. The inset shows the spectral changes. The main figure shows the absorbance change at 410 nm versus time (blue line). The solid lines represent the best fit line for single exponential decay. b) Rate constant versus hydrogen peroxide concentration for all variants tested. The solid lines represent the best fit line. Error bars represent ± SD, $n = 2$.

Fe(III)-Met80 695 charge transfer band measurement and background subtraction

A charge transfer band centred around 695 nm is indicative of Met80 coordination to the ferric heme. This transition is relatively weak and therefore high concentrations of cytochrome c (250 μM) need to be used to observe any changes. Upon oxidation by peroxide, the background absorbance changed quite dramatically and therefore to quantify changes in absorption, the background was removed. A fourth order polynomial was used to model the background absorbance and then subtracted. An example is given below. The black trace is the spectrum of WT cytochrome c (250 μM) between 600-800 nm at pH 5.4. The red trace is the background absorbance determined using an equation of the form $y = ax^4 + bx^3 + cx^2 + d$

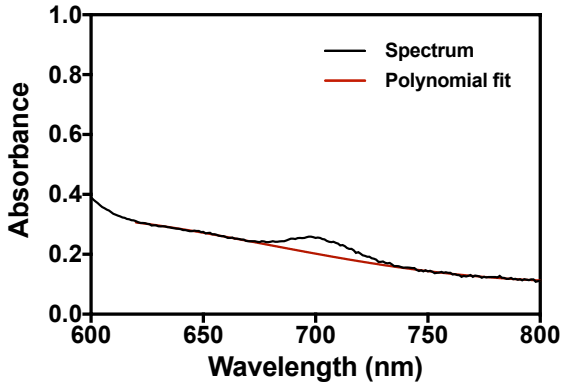

Figure S2. Example of how the background was fitted to determine accurately changes in the Fe(III)-Met80 absorbance at 695 nm.

Fe(III)-Met80 695 charge transfer band during the peroxidase reaction

Met80 coordination was investigated before and after the peroxidase reaction. Cytochrome c (250 μM), hydrogen peroxide (1 mM) and TMB (3 mM) were reacted at pH 5.4 for 60 minutes. Spectra were taken before and after. The far more intense Soret band was also measured by making a 1 in 10 dilution.

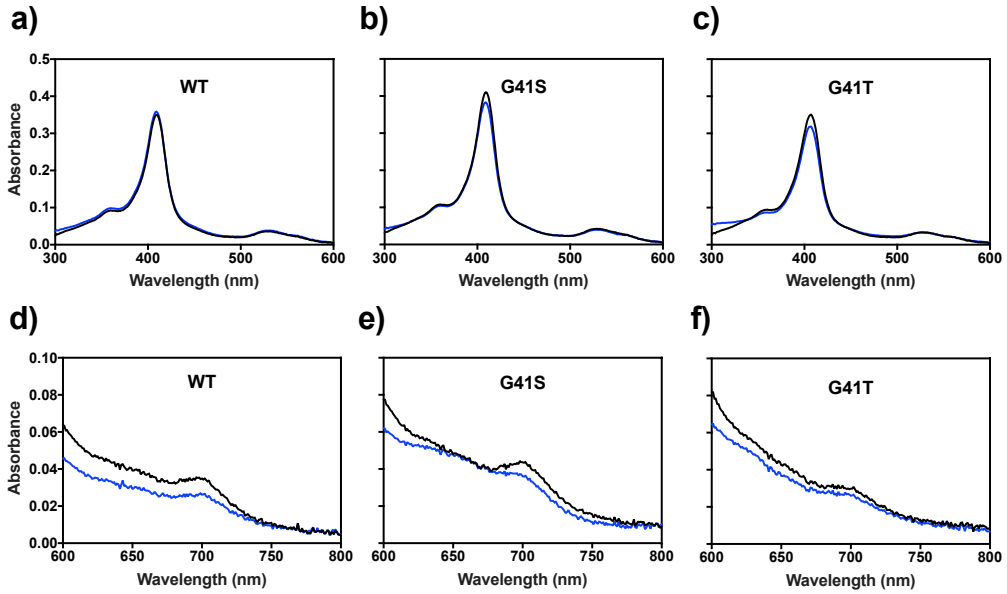

Figure S3. Spectral changes before (black line) and 60 min after (blue line) the peroxidase reaction was initiated. The Soret band (a-c) was measured by taking a 1:10 dilution. The 695 nm charge transfer band (d-f) is still visible after 60 minutes.

Azide binding to cytochrome c

Access to the heme iron was investigated by studying azide binding to all three variants of cytochrome c. Exogenous ligand binding to cytochrome c can be used as a diagnostic probe to assess the strength of the Met80 ligand. Azide forms stable complexes with the ferric heme and have been used extensively to probe the accessibility of the sixth coordination site of iron in heme proteins.[3] Upon binding of azide (N_3^-) to cytochrome c the following changes in the electronic spectrum are observed: a shift of the Soret maximum to 412 nm and an increase in intensity, a shift in the β band from 530 to 540 nm, and loss of the 695 nm band (Figure S4).

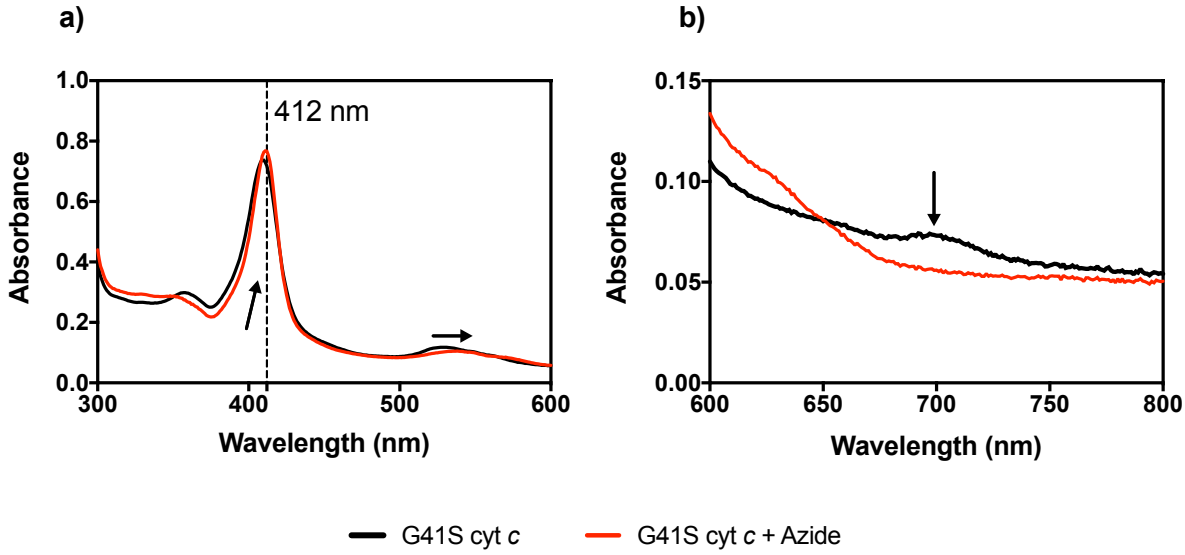

Figure S4. Changes in the absorption spectrum of cytochrome c upon binding with azide (1M) at pH = 5.4. a). Spectra generated between 300-600 nm upon mixing G41S cytochrome c (5 μM) in the absence (black trace) and presence (red trace) of azide. b). The visible spectrum between 600-800 nm of G41S cytochrome c (148 μM) in the absence (black trace) and presence (red trace) of azide.

The absorbance change at 412 nm upon addition of azide to cytochrome c variants was fitted using the following reaction scheme and equation (SE1). The fits are plotted in Figure S5 and the fitted apparent dissociation constants (K_{app}) given below.

$$\text{cyt c} - N_3^- \rightleftarrows \text{cyt c} + N_3^- \qquad K_{app} = \frac{[cyt\ c][N_3^3]}{[cytc{-}N_3^3]}$$

$$\Delta A_{412} = \varepsilon\{0.5\big(K_{app} + [cytc] + [azide]\big) - \sqrt{0.25(K_{app} + [cytc] + [azide])^2 - [cytc][azide]} \qquad \text{(SE1)}$$

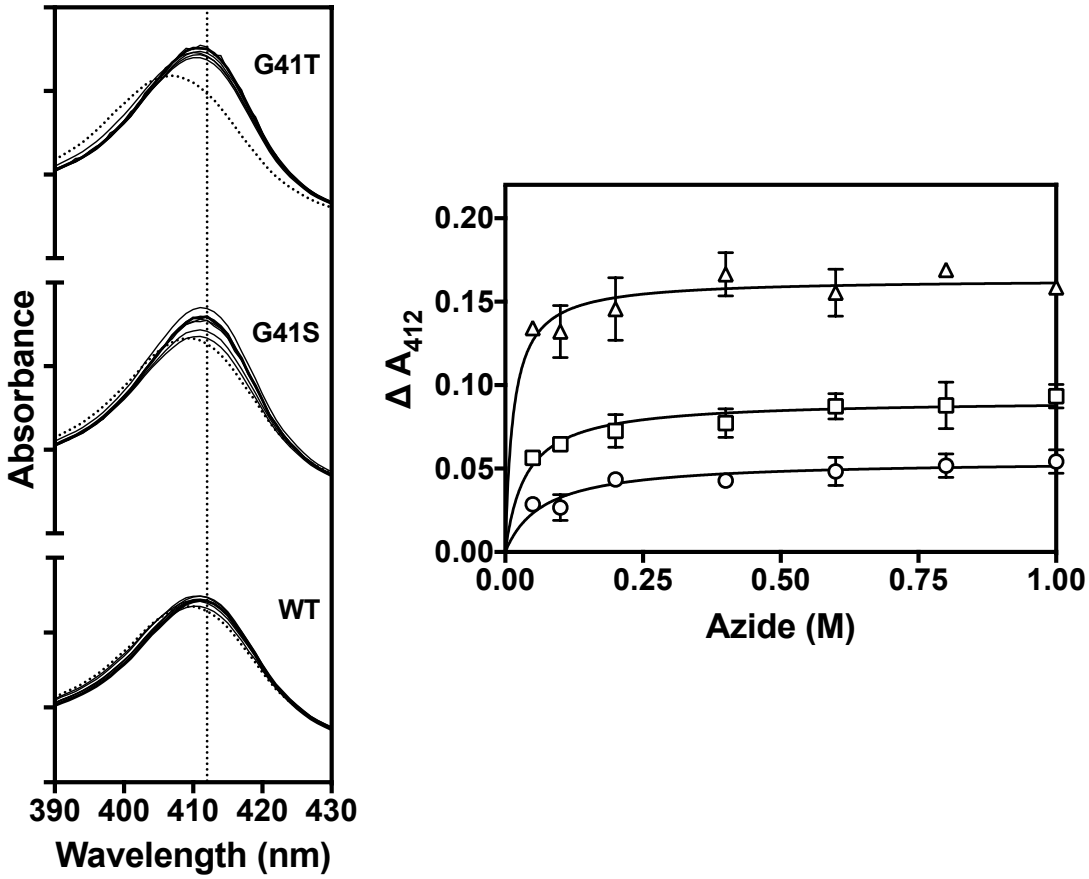

Figure S5. Quantification of azide-binding to cytochrome c variants (5 μM) at pH = 5.4. Changes in the soret band were followed as the azide concentration was altered from 0 M (dotted) to 1 M (solid) (left). The change in absorption was plotted and fitted to ascertain the apparent binding constant.

The following apparent dissociation constants (M) were obtained.

WT = 0.07 G41S = 0.04 G41T = 0.02

Direct infusion nanospray-coupled Orbitrap mass spectrometry

Mass spectrometry was used to probe the intact mass of cytochrome c during oxidation and the peroxidase reaction to show the protective effect of the substrate during the peroxidase reaction.

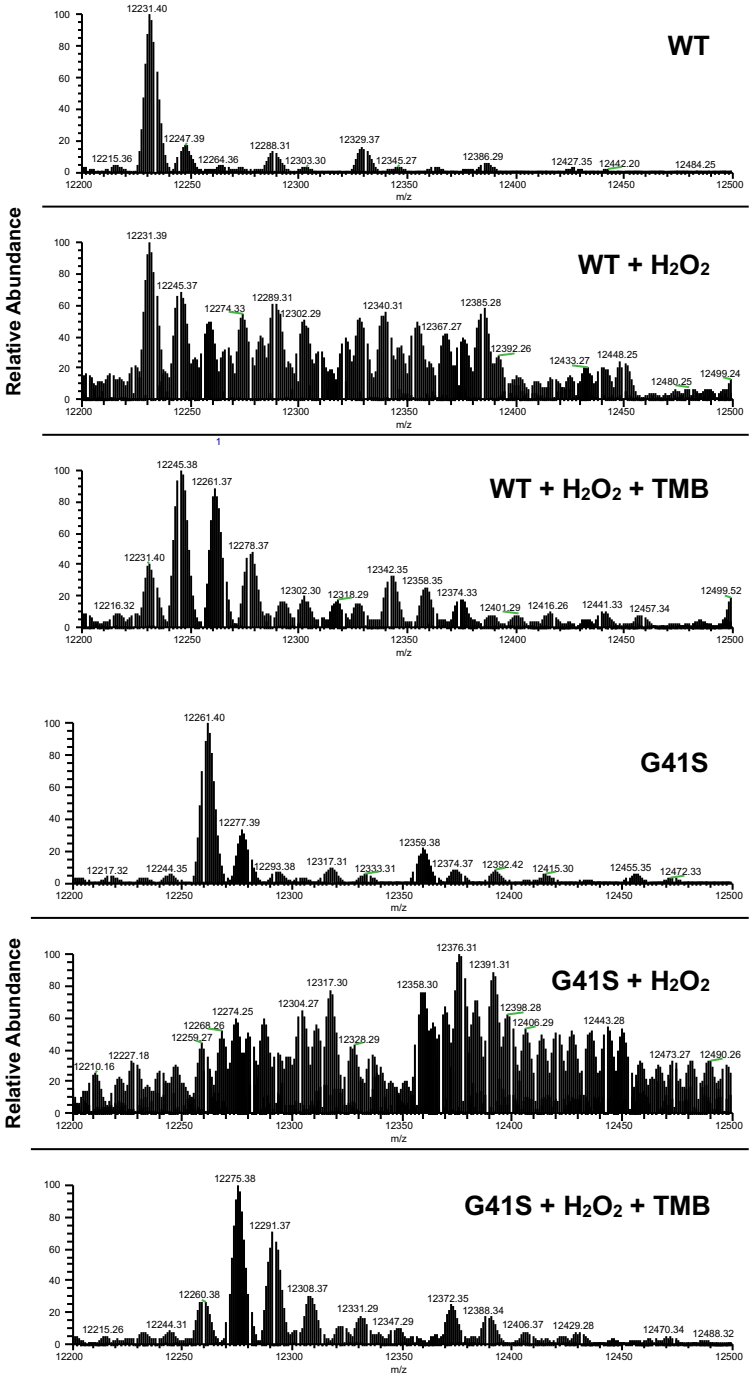

Figure S6. Intact mass-spectrometry of cytochrome c variants (5 μM) at pH = 5.4 either alone, in the presence of 100 μM hydrogen peroxide or in the presence of 100 μM peroxide and 1.6 mM TMB.

Mass spectrometric confirmation of Met80 oxidation

Collision-induced dissociation tandem mass spectrometry (MS/MS) was carried out to localize oxidative modifications in cytochrome c after H_2O_2 treatment. LC-coupled nanospray LTQ-Orbitrap MS/MS of tryptically digested cytochrome c was performed as outlined in experimental methods of the main text. The fragment ion spectra were analyzed by both software-based and manual data interpretation to pinpoint the sites of oxidative modifications.

The methionine 80 sulfur was specifically oxidized to sulfoxide and sulfone upon H_2O_2 treatment. The fragment ion spectra of the Met80 containing peptide M_{80}IFVGIK show a shift of +16 and +32 mass units for the Met and Ile containing b2-ions of the mono- and di-oxidised peptides respectively (Figure S7 & Table S1). The strong neutral loss peak of -64 mass units (CH_3SOH) from the mono-oxidized precursor ion (-32 *m/z* units from the doubly charged precursor ion at *m/z* 412.24078) further confirms the localization of oxidation on the methionine sulfur.

The extracted ion chromatograms of the unmodified, mono- and di-oxidized M_{80}IFVGIK peptides show a good chromatographic separation of all three molecular species which allows for the quantification of peptide intensities using the measurement of the area under the curve (Figure S8). We observed some in-source oxidation of the methionine sulfur, which is indicated by the detection of the mono- and di-oxidized forms at the retention time of the unmodified peptide. In-source oxidation is a common artefact in nanospray ionization at very low flow rates when no spray gas is used, causing desolvation of electrospray droplets in the atmospheric pressure region of the source.[2] The products of in-source oxidation were excluded from the quantification of Met80 oxidation.

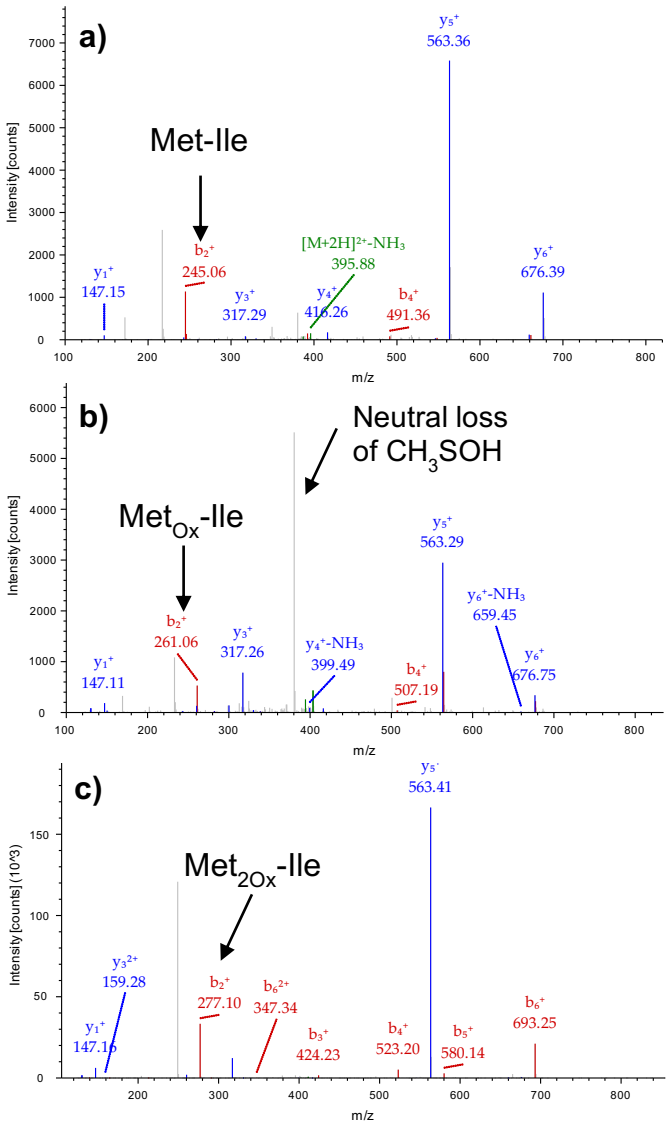

Figure S7. Collision-induced dissociation fragment ion spectra of the unmodified a), mono-oxidized b) and di-oxidized c) Met80 containing peptide M_{80}IFVGIK. The b-2 fragment ions (indicated by arrows), which contain the amino acid side chains of Met and Ile show a shift of +16 and +32 mass units in the spectrum of the mono- and di-oxidized peptides respectively. This and the neutral loss of CH_3SOH from the precursor ion of the mono-oxidized peptide b) clearly confirm oxidation of Met80.

Table S1. Software-based (Sequest HT) assignment of sequence ions in the MS/MS spectra of unmodified a), mono-oxidized b) and di-oxidized c) M_{80}IFVGIK. The detected ions are shown in red (b-ions which contain the N-terminus) and blue (y-ions which contain the C-terminus).

#1	**b^+**	**b^{2+}**	**sequence**	**y^+**	**y^{2+}**	**#2**
a) unmodified M_{80}IFVGIK						
1	132.04778	66.5273	M			7
2	245.13185	123.06956	I	676.43926	338.72327	6
3	392.20027	196.60377	F	563.35519	282.18123	5
4	491.26869	246.13798	V	416.28677	208.64702	4
5	548.29016	274.64872	G	317.21835	159.11281	3
6	661.37423	331.19075	I	260.19688	130.60208	2
7			K	147.11281	74.06004	1
b) mono-oxidized M_{80}IFVGIK						
1	148.04269	74.52498	M+O			7
2	261.12676	131.06702	I	676.43926	338.72327	6
3	408.19518	204.60123	F	563.35519	282.18123	5
4	507.26360	254.13544	V	416.28677	208.64702	4
5	564.28507	282.64617	G	317.21835	159.11281	3
6	677.36914	339.18821	I	260.19688	130.60208	2
7			K	147.11281	74.06004	1
c) di-oxidized M_{80}IFVGIK						
1	164.03761	82.52244	M+2O			7
2	277.12168	139.06448	I	676.43926	338.72327	6
3	424.19010	212.59869	F	563.35519	282.18123	5
4	523.25852	262.13290	V	416.28677	208.64702	4
5	580.27999	290.64363	G	317.21835	159.11281	3
6	693.36406	347.18567	I	260.19688	130.60208	2
7			K	147.11281	74.06004	1

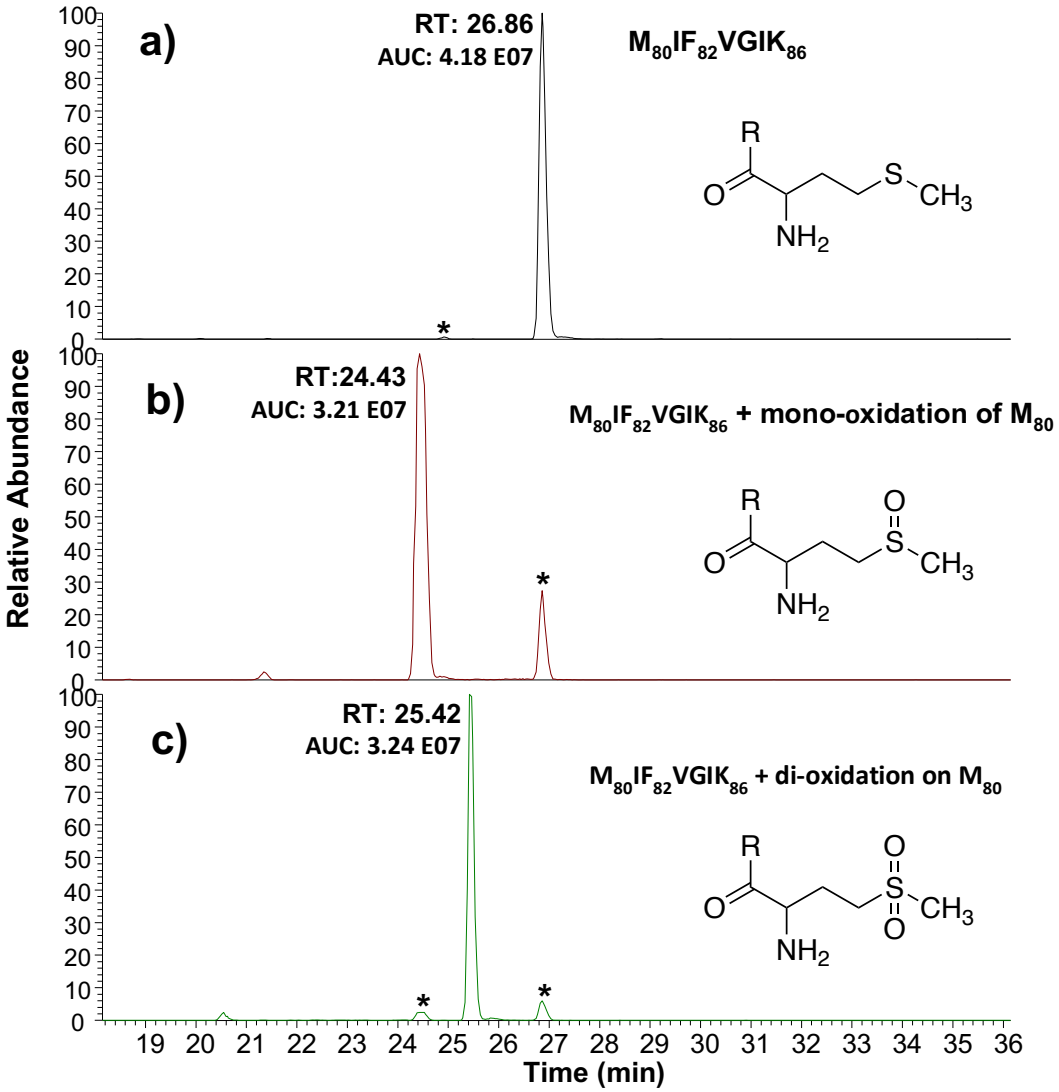

Figure S8. Extracted ion chromatograms of the unmodified a), mono-oxidized b) and di-oxidized c) Met80 containing M_{80}IFVGIK peptide. The area under the curve (AUC) is given for the relevant peaks at the indicated retention time (RT). The peaks indicated by asterisks (*) show the products caused by in-source oxidation,[2] which were not considered for quantification.

Quantification of peptides during the peroxidase reaction after cyanogen bromide digestion

To confirm the sensitivity of Met80 to oxidation during peroxidation, the reaction was repeated twice for each variant and then quantified by HPLC.

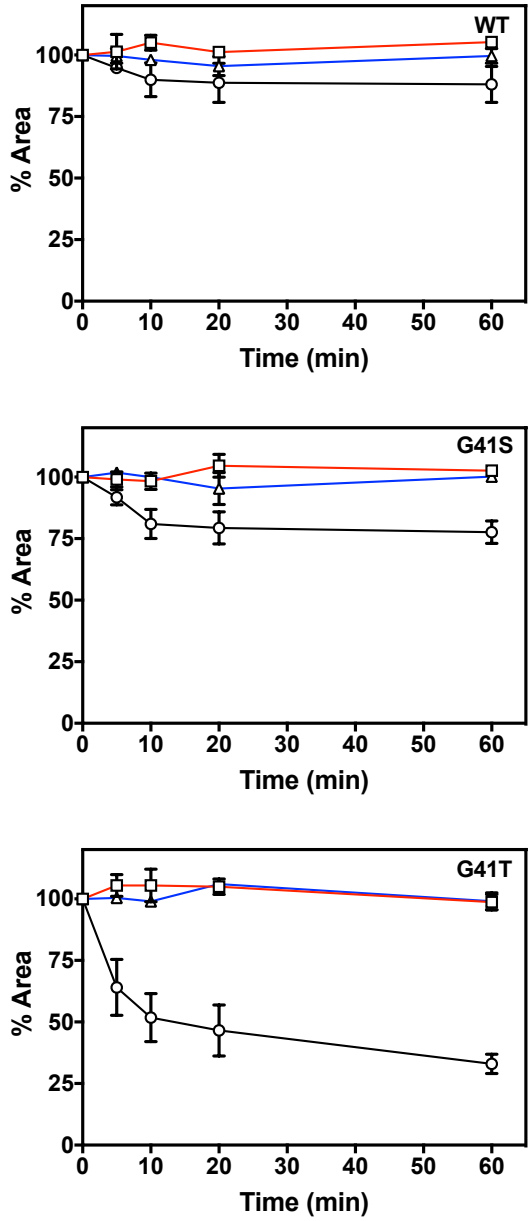

Figure S9. Cytochrome c (32 μM) was reacted with H_2O_2 (640 μM) and TMB (1 mM). After a defined reaction time, the sample was digested with CNBr re-suspended and separated by HPLC. The Met80 (–○–) and Met12 (–△–) containing peptides were followed at 215 nm, while the heme (–□–) was followed at 410 nm. Error bars represent ± SD, *n*=2

Cytochrome c oxidation by chloramine T and subsequent re-purification

Chloramine T was used to selectively oxidize methionine to methionine sulfoxide. Cytochrome c has three methionine residues, Met12, Met65 and Met80. The first two lie on the surface of the protein. Cytochrome c was treated with chloramine T (5 equivalents) over 3 hours. The protein was then purified through a HiTrap SP Sepharose column by FPLC and monitored at 410 nm. Representative FPLC traces of WT cytochrome c and variants treated with chloramine T are given in Figure S9.

Chloramine T treated cytochrome c was found to have a blue shifted Soret band of 406 nm and absence of the 695 nm band as observed previously.[4] UV-vis spectra of WT cytochrome c and chloramine T treated protein are shown in Figure S11. WT, G41S and G41T variants show almost identical spectra after treatment. Protein was digested as described in the paper and LC-coupled mass spectrometry collected. Three peptides, containing the three methionine residues were measured. The proportion of un-modified and oxidized peptides are shown in Figure S10 and the detected peptide masses as well as mass differences between un-modified and oxidized peptides are provided in Table S2.

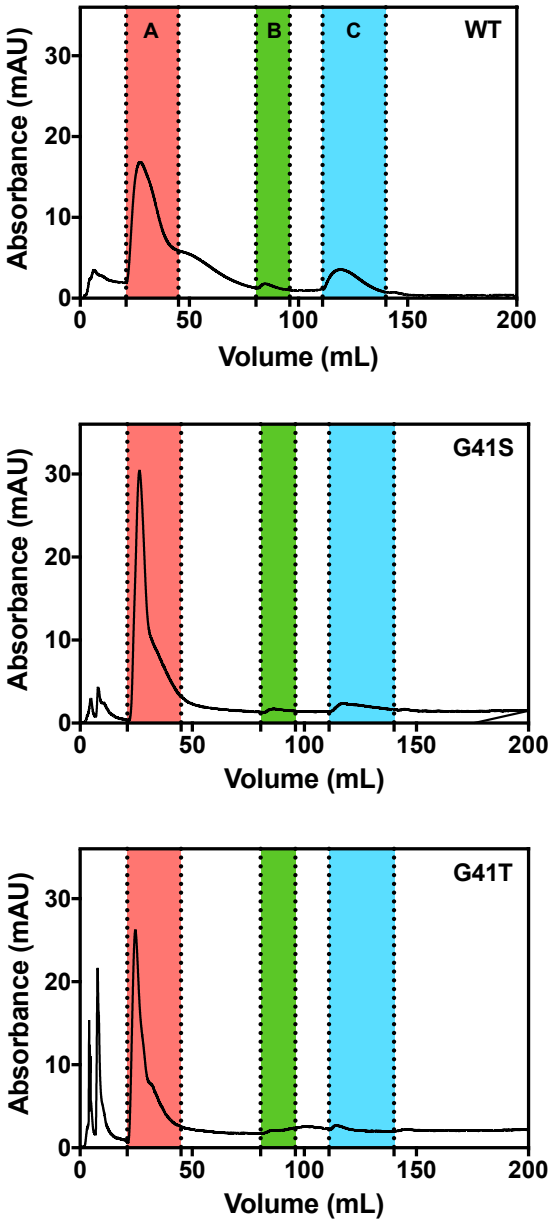

Figure S10. Representative FPLC trace showing purification of chloramine treated WT cytochrome c. Three fractions were collected (A, B, C) and mass spectra collected to determine the state of the methionine residues.

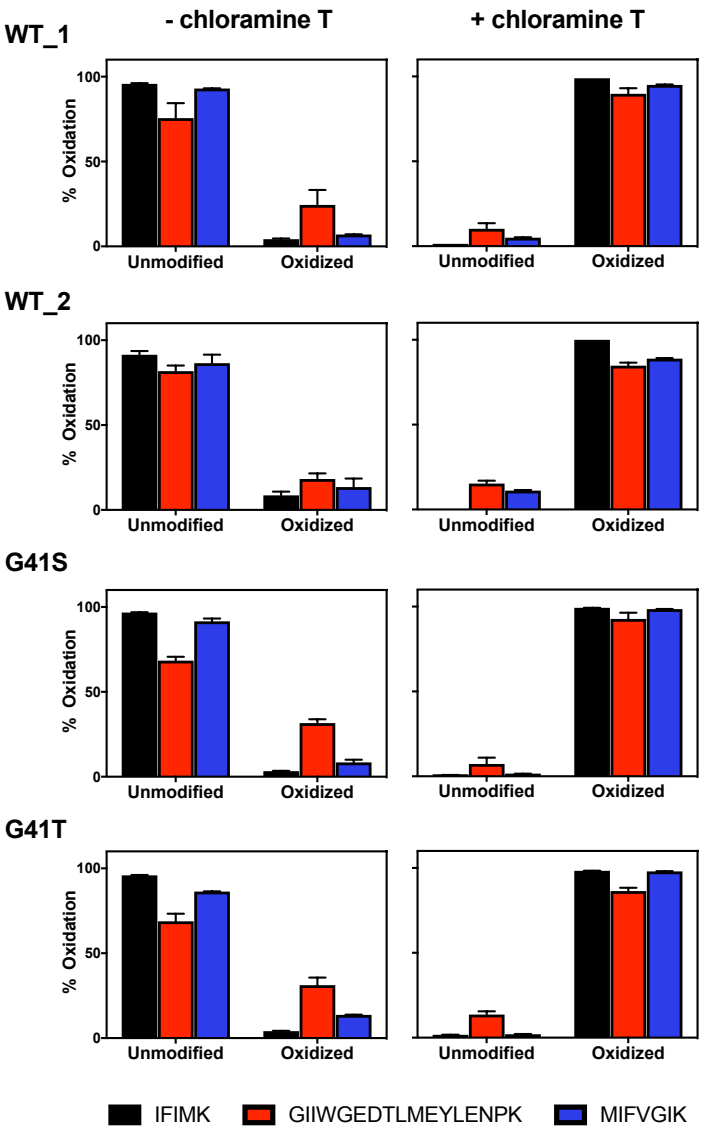

Figure S11. Chloramine-T treatment efficiently oxidizes the methionines in cytochrome c. The untreated and chloramine-T treated wildtype (WT_1 and WT_2), G41S and G41T cytochrome c from fraction A (see Figure S9) were digested with trypsin and the three tryptic peptides IFIMK (black), GIIWGEDTLMEYLENPK (red), and MIFVGIK (blue) were analyzed by LC-coupled Orbitrap MS to measure the efficiency of methionine oxidation. The area under the curve of the extracted ion chromatograms for the respective peptide signals was used to calculate the relative oxidation of each peptide in each sample. The wildtype cytochrome c was measured in two biological replicates (WT_1 and WT_2) and the mutants G41S and G41T were measured as single samples. Each sample was measured in three technical replicates (error bars). More than 80 % of each peptide becomes oxidized upon chloramine-T treatment. Collision-induced dissociation tandem mass spectrometry was used to confirm the site of oxidation on the methionine sulfur (data not shown).

Table S2. Accurate masses of methionine containing tryptic peptides of cytochrome c with and without chloramine-T treatment and the exact mass difference between the unmodified and oxidized form. (Taken from fraction A above, Figure S9).

		unmodified peptide	chloramine T treated		
Peptide sequence	Ion	Detected *m/z* (mass)	Detected *m/z* (mass)	Δ mass	Modified residue
IFIM$_{12}$K	$[M+H]^+$	651.3888 (650.3815)	667.3840 (666.3764)	+15.9949	Met12
GIIWGEDTLM$_{65}$EYLENPK	$[M+2H]^{2+}$	1004.4879 (2006.9610)	1012.4867 (2022.9592)	+15.9960	Met65
M$_{80}$IFVGIK	$[M+2H]^{2+}$ / $[M+H]^+$	404.2437 (806.4728)	823.4742 (822.4678)	+15.9950	Met80

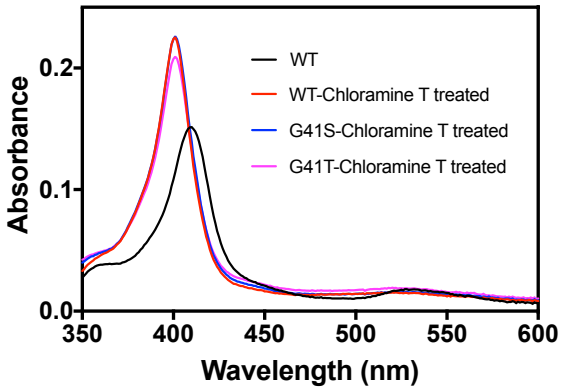

Figure S12. UV-vis spectra of WT cytochrome c and chloramine T treated variants showing the same shift in the Soret band after treatment.

Analysis of protein stability after chloramine T oxidation

Protein stability after chloramine T oxidation was analyzed using an assay based on SYPRO Orange dye.[5] Cytochrome c (5 μM final) was mixed with SYPRO orange dye (5x final) in 384-well plates and measured in a thermo-cycler (Roche light cycle 480 II). The fluorescence signal (490 nm excitation, 580 nm emission) was measured while the temperature was changed. Starting at 20°C, the temperature was increased to 85°C with a rate of 10°C/min and then decreased again to 20°C. The experiment was repeated three times with different solutions and the data fitted to the following equation.

$$fluoresence = constant + \frac{fluoresence_{max} - constant}{(1+e^{\left(\frac{T_m - T}{slope}\right)})} \quad \text{(SE2)}$$

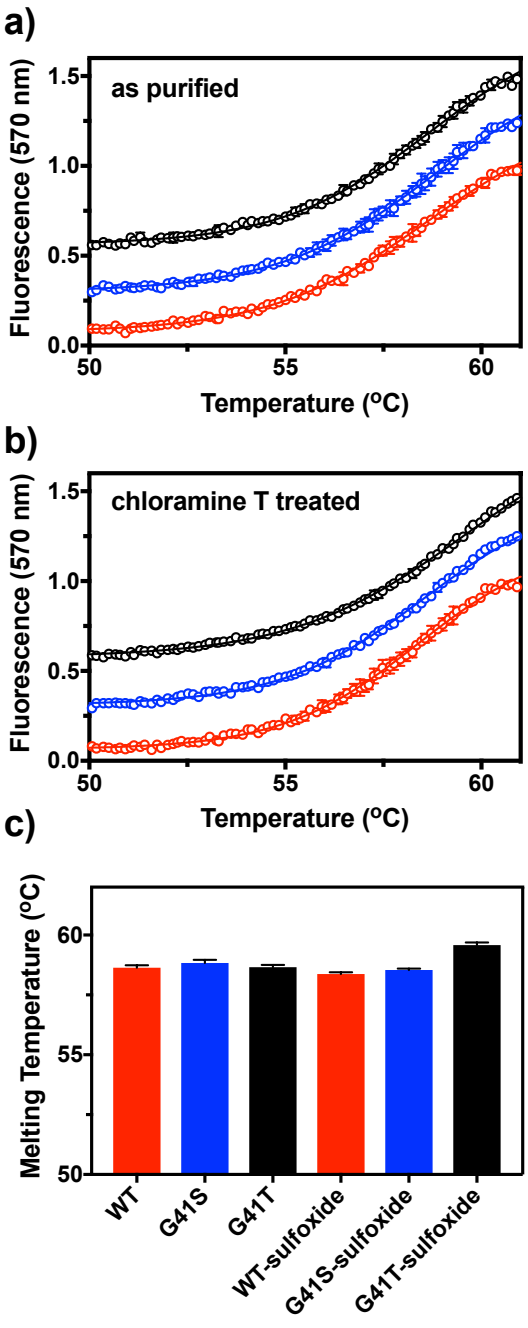

Figure S13. Analysis of protein stability before and after chloramine T oxidation using SYPRO Orange dye. a) Fluorescent emission at 570 nm during denaturation of WT (red), G41S (blue) and G41T (black). The solid line is a non-linear fit using equation SE2. The three curves have been separated in the *y* axis to aid clarity. b) Similar denaturation curves for WT, G41S and G41T after treatment with chloramine T. c) plot of the melting temperature determined through non-linear fitting. Error bars represent ± SD, $n = 3$.

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