Supplementary data for the article:

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Supporting Information

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Influence of methionine residue position on oxidative stability of glucose oxidase from *Aspergillus niger*

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Reagents. Yeast nitrogen base and casamino acids were obtained from Formedium (Norfolk, UK). ABTS and HRP were obtained from AppliChem (Darmstadt, Germany). All other reagents were obtained from Sigma Aldrich (St. Luis, MO).

Saturation mutagenesis of glucose oxidase at different methionine positions. Saturation libraries were created using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) and *Escherichia coli* XL10gold. We designed 14 primers (Eurofins MWG Operon) and made libraries using wt-GOx (housed in pCTCON2 vector) as a template. The primer sequences are given below (where NNN represents each of the 64 codons).

Met190	FP	CGTCAAGGCTCTCNNNAGCGCTGTCGAAG
	RP	CTTCGACAGCGCTNNNGAGAGCCTTGACG
Met214	FP	CATGGTGTGTCCNNNTTCCCCAACACC
	RP	GGTGTTGGGGAANNNGGACACACCATG
Met305	FP	CCGGTATCGGANNNAAGTCCATCCTG
	RP	CAGGATGGACTTNNNTCCGATACCGG
Met480	FP	CTCCGGTGCCNNNCAGACCTACTTC
	RP	GAAGTAGGTCTGNNNGGCACCGGAG
Met523	FP	GGTACTTGCTCCNNNATGCCGAAGGAG
	RP	CTCCTTCGGCATNNNGGAGCAAGTACC
Met524	FP	CTTGCTCCATGNNNCCGAAGGAGATG

	RP	CATCTCCTTCGGNNNCATGGAGCAAG
Met528	FP	GCCGAAGGAGNNNGGCGGTGTTG
	RP	CAACACCGCCNNNCTCCTTCGGC
Met556	FP	CCTCCTACGCAANNNTCGTCCCATGTCATG
	RP	CATGACATGGGACGANNNTTGCGTAGGAGG
Met561	FP	CGTCCCATGTCNNNACGGTGTTCTATG
	RP	CATAGAACACCGTNNNGACATGGGACG
Met567	FP	GGTGTTCTATGCCNNNGCGCTAAAAATTTCGG
	RP	CCGAAATTTTTAGCGCNNNGGCATAGAACACC
Met582	FP	GATTATGCTTCCNNNCAGGGAGGATCCG
	RP	CGGATCCTCCCTGNNNGGAAGCATAATC

Cloning combinatorial mutants in *S. cerevisiae and P. pastoris.* Combinatorial mutants were made with primers carrying specific mutations using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) and *E. coli* Xl10gold. As template for the mutagenesis, we used wtGOx housed in pCTCON2 and pPICZαA vectors (Invitrogen). The primer sequences are given below.

M214T	FP	CATGGTGTGTCCACCTTCCCCAACACC
	RP	GGTGTTGGGGAAGGTGGACACACCATG

M556L	FP	CCTCCTACGCAACTATCGTCCCATGTCATG
	RP	CATGACATGGGACGATAGTTGCGTAGGAGG
M561S	FP	CGTCCCATGTCAGTACGGTGTTCTATG
	RP	CATAGAACACCGTACTGACATGGGACG
M567F	FP	GTGTTCTATGCCTTTGCGCTAAAAATTTCG
	RP	CGAAATTTTTAGCGCAAAGGCATAGAACAC

Expression of the GOx variants in *P. pastoris* KM71H. Cells from the agar plates were inoculated into 10 mL BMGH (100 mM potassium phosphate (pH 6.0), 1.34% (w/v) yeast nitrogen base with ammonium sulfate (YNB), 1.61 μM biotin, 1% (v/v) glycerol) medium and incubated for 24 h at 30 °C, 200 rpm. The initial culture was transferred into 1 L BMGH medium followed by the incubation for additional 24 h. After centrifugation at 3,000 g for 10 min, cells were transferred to 200 mL BMMH (identical to BMGH, except for 0.5% (v/v) methanol instead of 1% (v/v) glycerol) medium and incubated at 27 °C, 200 rpm, for 4 days. Every 24 h, 50% (v/v) methanol solution was added to the fermentation medium up to the desired final methanol concentration of 0.5% (v/v).

SUPPLEMENTAL RESULTS

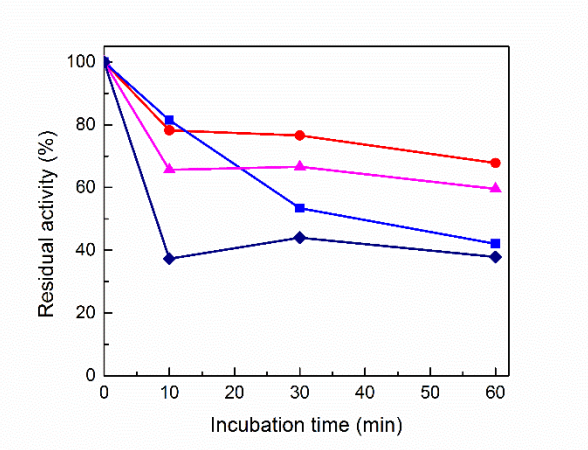

Figure S1. Inactivation of wt-GOx using:: (●) 50 mM H_2O_2 without glucose,(▲) 500 mM H_2O_2 without glucose, (■) 50 mM H_2O_2 with 100 mM glucose added, (♦), 500 mM H_2O_2 with 100 mM glucose added.

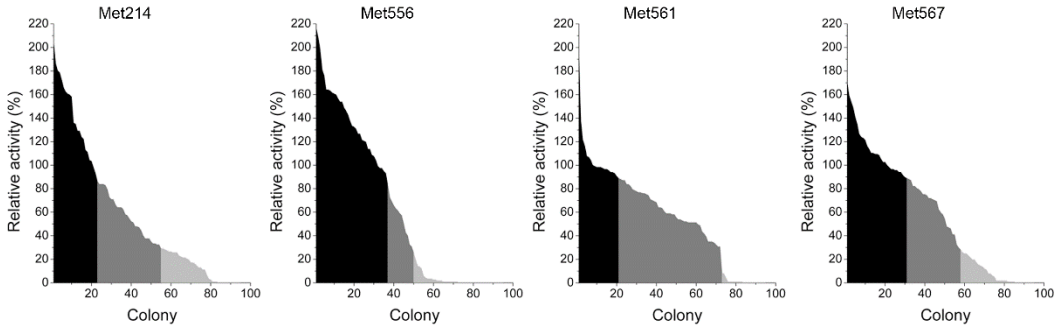

Fig. S2. Activity screening of the four saturation libraries at the methionine positions with the most significant effect on GOx oxidative stability, where colonies are sorted in the descending order based on the relative activity compared to the wt-GOx activity, and assigned to one of the three groups: activity ≥ 90% (black), 90 % < activity ≥ 30 % (dark grey), and activity < 30% of the wt-GOx (light grey).

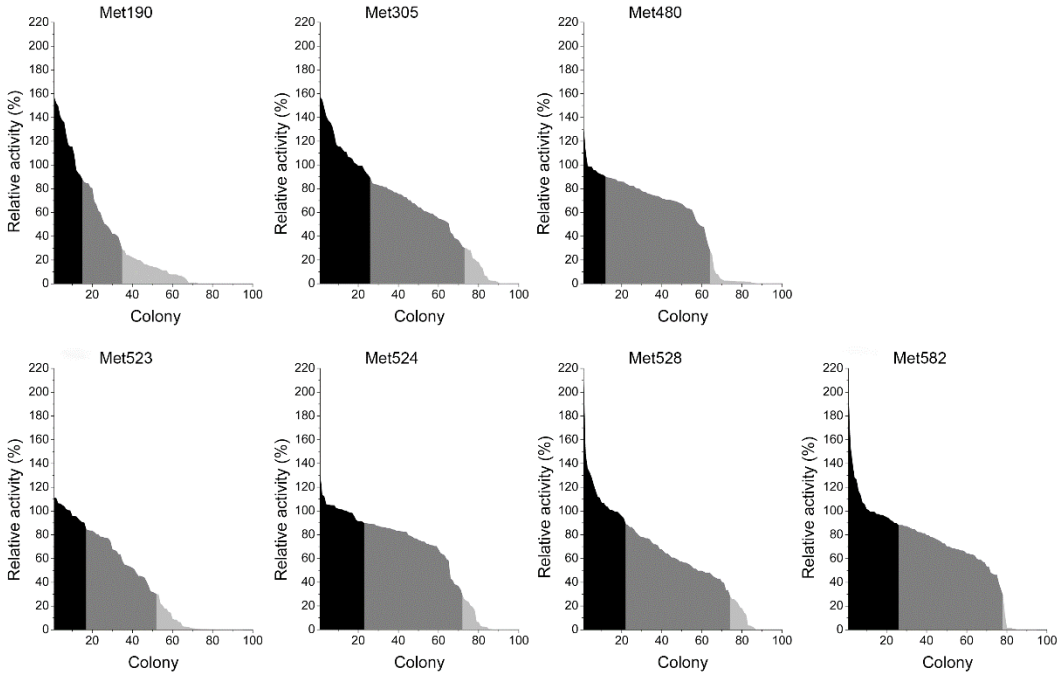

Figure S3. Activity screening of the seven saturation libraries at the positions with no significant effect on GOx stability, where colonies are sorted in the descending order based on the relative activity compared to the wt-GOx activity, and assigned to one of the three groups: activity ≥ 90% (black), 90 % < activity ≥ 30 % (dark grey), and activity < 30% of the wt-GOx (light grey).

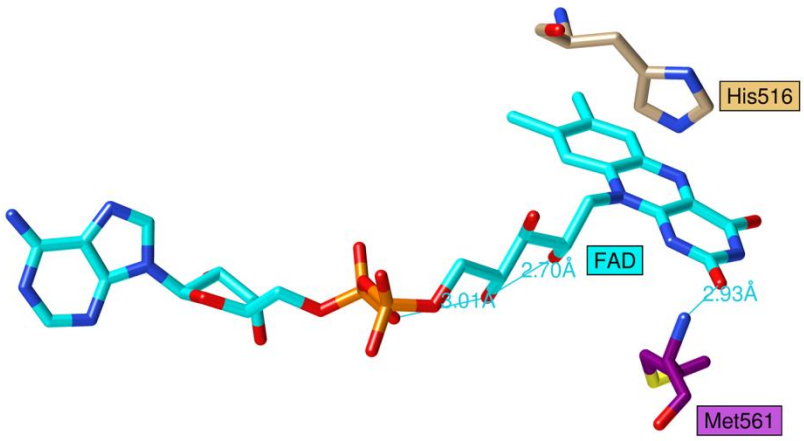

Figure S4. Graphical representation of Met561 position relative to the FAD cofactor and His516 in GOx (PDB: 1CF3)

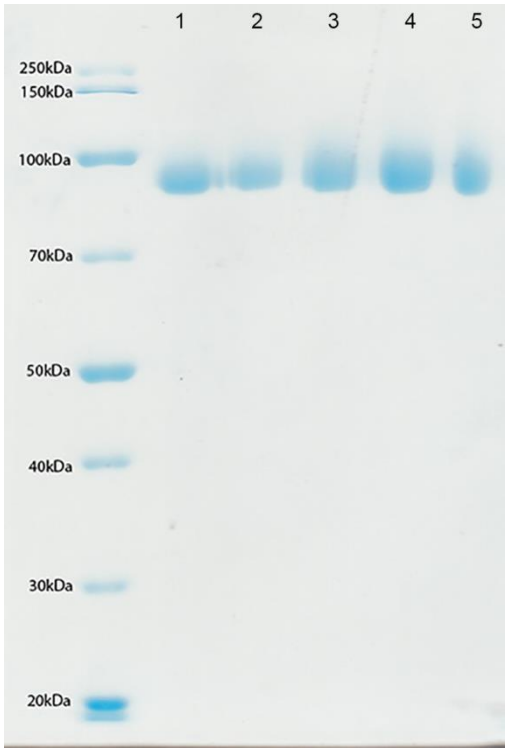

Figure S5. SDS-PAGE of the purified GOx variants; 1) wt-GOx; 2) M214T-GOx; 3) M561S-GOx; 4) M214T/M556L-GOx; 5) M561S/M556L-GOx.

Table S1. Residual activities of the best mutants discovered in the saturation libraries at the positions of Met190, Met 305, Met 480, Met 524, Met528, Met582 (second and third group). The activity was measured before (non-oxidized) and after (oxidized) hydrogen peroxide treatment. The residual activity represents the ratio of the oxidized and non-oxidized activities. Values are reported as average ± standard deviation of three independent experiments

GOx variant			**Activity**		
Met position	**Library label**	**Mutation**	**Non-oxidized (U mL^{-1})**	**Oxidized (U mL^{-1})**	**Residual (%)**
WT	—	—	0.117 ± 0.020	0.053 ± 0.008	46.3 ± 6.8
190	C5	M190I	0.074 ± 0.006	0.048 ± 0.006	64.8 ± 8.1
305	B3	M305C	0.127 ± 0.004	0.061 ± 0.012	48.2 ± 9.4
	F10	M305R	0.132 ± 0.002	0.063 ± 0.008	47.8 ± 6.1
480	D2	M480L	0.139 ± 0.005	0.059 ± 0.022	42.6 ± 15.8
524	H6	M524C	0.073 ± 0.001	0.040 ± 0.002	55.3 ± 2.7
528	C10	M528A	0.081 ± 0.013	0.053 ± 0.004	65.4 ± 4.9
	E11	M528I	0.102 ± 0.020	0.046 ± 0.003	45.1 ± 2.9
582	C6	M582A	0.081 ± 0.006	0.052 ± 0.001	64.2 ± 1.2
	F6	M582T	0.094 ± 0.006	0.048 ± 0.001	51.1 ± 1.1

Table S2. Residual activities of the most promising mutants discovered in the saturation libraries at the positions of Met214, Met561, Met556 and Met567. The activity was measured before (non-oxidized) and after (oxidized) treatment with 50 mM hydrogen peroxide in presence of 100 mM glucose at pH 5.5. The residual activity represents the ratio of the oxidized and non-oxidized activities. Values are reported as average ± standard deviation of three independent experiments.

GOx variant			**Activity**		
Met position	**Library label**	**Mutation**	**Non-oxidized (U mL^{-1})**	**Oxidized (U mL^{-1})**	**Residual (%)**
WT	—	—	0.117 ± 0.020	0.053 ± 0.008	46.3 ± 6.8
214	A3, B7, E4[a]	**M214T**	**0.166 ± 0.011**	0.091 ± 0.007	**54.8 ± 4.2**
	D10	M214C	0.185 ± 0.011	0.075 ± 0.005	40.5 ± 2.7
556	D4	**M556L**	**0.126 ± 0.005**	**0.075 ± 0.005**	**59.4 ± 4.0**
	A5	M556T	0.110 ± 0.001	0.054 ± 0.002	49.0 ± 1.8
	B8, C2[b]	M556I	0.153 ± 0.007	0.074 ± 0.005	48.3 ± 3.2
561	F4	M561A	0.070 ± 0.007	0.051 ± 0.005	72.8 ± 7.1
	G1	M561T	0.081 ± 0.008	0.057 ± 0.006	70.4 ± 7.4
	B2, F7, H4[a]	**M561S**	**0.101 ± 0.007**	0.071 ± 0.002	**70.2 ± 2.0**
	B9, E12, H11[a]	M561G	0.117 ± 0.007	0.078 ± 0.004	66.7 ± 3.4
567	E6, E12[b]	**M567F**	**0.066 ± 0.004**	0.043 ± 0.003	**65.1 ± 4.5**
	C3	M567T	0.075 ± 0.004	0.045 ± 0.007	59.5 ± 9.3

[a] Average activities over the three samples found bearing the same mutation.

[b] Average activities over the two samples found bearing the same mutation.

Table S3. Residual activities of the combinatorial GOx mutants. The activity was measured before (non-oxidized) and after (oxidized) hydrogen peroxide treatment. The activity was measured before (non-oxidized) and after (oxidized) treatment with 50 mM hydrogen peroxide in presence of 100 mM glucose at pH 5.5. The residual activity represents the ratio of the oxidized and non-oxidized activities. Values are reported as average ± standard deviation of three independent experiments.

GOx variant	**Activity**		
	Non-oxidized ($U\ mL^{-1}$)	**Oxidized ($U\ mL^{-1}$)**	**Residual (%)**
WT	0.488±0.005	0.199±0.023	**40.8±4.7**
M214T/M556L	0.747±0.030	0.292±0.034	**39.1±4.6**
M214T/M561S	0.373±0.026	0.218±0.007	**58.6±1.9**
M214T/M567F	0.188±0.010	0.055±0.001	**29.1±0.7**
M561S/M556L	0.570±0.018	0.371±0.035	**65.0±6.2**
M561S/M567F	0.574±0.010	0.265±0.019	**46.2±3.2**
M567F/M556L	0.448±0.025	0.142±0.045	**31.6±10.1**
M214T/M561S/M556L	0.214±0.024	0.110±0.003	**51.5±1.6**
M214T/M561S/M567F	ND	ND	**ND**
M214T/M556L/M567F	0.229±0.017	0.155±0.017	**67.7±7.3**
M561S/M556L/M567F	0.439±0.092	0.263±0.038	**59.9±8.7**

Table S4. Residual activities of the GOx expressed in *S. cerevisaie* (YSD) and immobilized on the macroporous poly(glycidyl methacrylate) polymer. The activity was measured before and after treatment with 50 mM hydrogen peroxide in presence of 100 mM glucose at pH 5.5. The residual activity represents the ratio of the oxidized and non-oxidized activities. Values are reported as average ± standard deviation of three independent experiments. Half-life of oxidative stability was calculated from residual activity of purified GOx mutants and wild-type enzyme immobilized on macroporous poly(glycidyl methacrylate) polymer using equation for exponential first-order kinetics ($A=A_oe^{-kt}$).

GOx variant	**Residual activity (%)**		**$t_{1/2}$ (min)**
	YSD	**Immobilized**	
WT	46.3 ± 6.8	43.7 ± 5.5	50.2±5.2
M214T	54.8 ± 4.2	57.5 ± 4.2	75.1±3.0
M561S	70.2 ± 2.0	71.3 ± 8.5	122.9±5.0
M214T/M556L	39.1±4.6	42.8±0.6	49.0±0.6
M561S/M556L	65.0±6.2	63.8±6.8	92.5±4.4