Supplementary Table S1.

X-ray data collection and refinement statistics of T41L/N71S and T41Q/N71S/F124T NfsB with and without bound nicotinate.

Protein	**T41L/N71S**	**T41L/N71S with nicotinate**	**T41Q/N71S/F124T**	**T41Q/N71S/F124T with nicotinate**
PDB code	**8C5P**	**8CCV**	**8C5F**	**8C5E**
Data collection				
Wavelength (Å)	0.934	0.934	0.934	1.00
Resolution range (Å)	43.16-1.69 (1.74-1.69)	47.75-2.20 (2.26- 2.20)	47.85-1.60 (1.69-1.60)	43.5-1.65 (1.70-1.65)
Space group	P 4_12_12	P 4_12_12	P 4_12_12	P 4_12_12
Unit cell	57.2 Å, 57.2 Å, 263 Å 90°, 90°, 90°	56.9 Å, 56.9 Å, 264 Å 90°, 90°, 90°	57.2 Å, 57.2 Å, 262 Å 90°, 90°, 90°	57.6 Å, 57.6 Å, 264 Å 90°, 90°, 90°
Total reflections	647,523 (11,240)	172,983 (10,839)	345,900 (37,708)	290,689 (18,810)
Unique reflections	49,521 (3,011)	15,934 (1,068)	57,801 (8,552)	53,617 (6,747)
Multiplicity	13.1 (3.54)	10.9 (10.1)	5.9 (4.41)	5.4, (2.79)
Completeness (%)	99.0 (87.5)	96.5 (77.1)	98.6 (92.3)	97.3 (81.4)
$\langle I/\sigma I \rangle$ overall	36.9 (3.4)	24.9 (5.2)	27.2 (5.1)	13.2 (1.9)

Mosaicity	0.096	0.214	0.112	0.124
Wilson B-factor (Å^2)	15.1	39.9	14.1	17.8
Anisotropy	0.339	0.46	0.071	0.044
Rsym[1] (%)	5.0 (34.8)	7.5 (53.2)	4.5 (29.3)	7.8 (55.7)
Refinement and quality				
Reflections used in refinement	47,036	22,344	57,878	53,648
Reflections used for R-free	2,499 (5.0%)	1,138 (5.1%)	2,896 (5.0%)	2,751 (5.15 %)
R-work[2]	0.150	0.185	0.158	0.176
R-free[3]	0.188	0.243	0.181	0.217
Number of non-hydrogen atoms				
macromolecules	3,485	3,352	3,418	3,402
ligands	114	80	114	84
solvent	545	167	500	517
Protein residues	432	432	432	432
RMSD bond length (Å)	0.015	0.010	0.014	0.006

RMSD bond angles (°)	1.82	1.70	1.52	1.03
RMSD chirality	0.102	0.087	0.101	0.079
RMSD planarity	0.014	0.010	0.010	0.004
F_o-F_c correlation	0.97	0.96	0.96	0.94
Ramachandran plot				
Ramachandran favored (%)	99	98	99.5	100
Ramachandran allowed (%)	1	2	0.5	0
Ramachandran outliers (%)	0	0	0	0
Sidechain outliers (%)	0.8	2	0.6	0.8
All atom clashscore	3	1	2	1
RSRZ outliers (%)	0.2	2.5	0	2.3
Average B-factors (Å^2)	19.0	55	19.0	13.0
macromolecules	16	52	16	10
ligands	33.6	45.2	33.6	15.4
FMN	10	39.5	10	13

Nicotinamide	-	65	-	20.5
solvent	50.7	49.6	58.1	55.2

- The numbers in parentheses represent statistics in the highest resolution shell.
- *1.* $R_{sym} = \Sigma|I_i - <|>|/\Sigma|I_i|$ where I_i is the intensity of the *i*th measurement, and <|>is the mean intensity for that reflection;
- *2.*$R_{work} = \sum\|F_o| - |F_c|/\sum|F_o|$, where F_o and F_c are the observed and calculated structure factors for data used for refinement, respectively.
- *3.* $R_{free} = \sum\|F_o| - |F_c|/\sum|F_o|$ for 5% of the data not used at any stage of structural refinement.

Supplementary Table S2:

Redox potentials of WT NfsB and mutants.

Titrations were performed with 50-100 μM protein in 50 mM phosphate buffer, pH 7.5, 500 mM KCl, 10% glycerol, in the absence of redox mediators, with two aliquots of the same enzyme preparation. The data was fitted to either 2 single electron transfer steps (equation 1) or a concerted 2 electron transfer step (equation 2) using Sigmaplot14 with equal weighting of all points**.**

Enzyme	**Fit to 2 single electron transfers**						**Fit to concerted electron transfer**			
	E_1 (mV)	**P**	**E_2 (mV)**	**P**	**Rsqr**	**Standard error**	**Em**	**P**	**Rsqr**	**Standard error**
WT	-231 ± 2.8	<0.0001	-204 ± 2.8	<0.0001	0.9975	0.0219	-217.8 ± 0.7	<0.0001	0.9924	0.0376
T41L/N71S	-215 ± 1.6	<0.0001	-252 ± 1.6	<0.0001	0.9968	0.0225	-233 ± 1.6	<0.0001	0.9635	0.0774
T41Q/N71S/F124T	-245 ± 2.6	<0.0001	-215 ± 2.6	<0.0001	0.9980	0.0194	-230 ± 0.7	<0.0001	0.9932	0.0354

Supplementary Table S3:

Stopped-flow reduction of WT and mutants by NADH and by CB1954.

For the NADH reactions solutions containing 10 μM enzyme in one syringe and 25-1000 μM NADH in the other syringe, both in 10 mM Tris pH 7, buffer were mixed rapidly and the absorbance at 340 nm was monitored with time. The pseudo first-order rate constants were obtained by fitting the data at each concentration of substrate to an exponential decay. These were then fitted to a hyperbola in Sigmaplot 14 to obtain the kinetic parameters below. The CB1954 results are from [1].

Enzyme	Reduction by NADH									CB1954 reduction from [1]	
	k (s^{-1})	t	P	K_d (μM)	t	P	k/K_d (μM^{-1} s^{-1})	t	P	k (s^{-1})	k/K_d (μM^{-1} s^{-1})
WT	1340 ± 84	15.8	<0.0001	220 ± 30	7.4	<0.0001	6.0 ± 0.5	12.6	<0.0001	> 30	0.029 ± 0.001
T41L/N71S	260 ± 11	25	<0.0001	160 ± 15	11	<0.0001	1.7 ± 0.1	17.3	<0.0001	> 550	1.6 ± 0.1
T41Q/N71S/F124T	290 ± 13	22	<0.0001	90 ± 12	8	<0.0001	3.0 ± 0.3	11.7	<0.0001	> 400	0.76 ± 0.02

1 Jarrom, D., Jaberipour, M., Guise, C. P., Daff, S., White, S. A., Searle, P. F. and Hyde, E. I. (2009) Steady-state and stopped-flow kinetic studies of three Escherichia coli NfsB mutants with enhanced activity for the prodrug CB1954. Biochemistry. **48**, 7665-7672

Supplementary Table S4:

Selection of quadruple mutations of NfsB

NfsB was mutated at 8 positions within the active site and the gene introduced into lambda phage. E coli lysogens were made and mutants were selected using the NfsB reduction of CB1954 to activate the SOS response and release phage enriched with more active enzyme. Amino-acid substitutions, frequency and prodrug sensitivity of mutants analysed after 15 rounds of selection:

T41	N71	G120	F124	M127	H128	W138	NUMBER	APPROX IC_{50}-REPLICA PLATE (μM CB1954)	IC_{50} colony assay (μM CB1954)
Q	S		T	V			3	<10	4.5
Q	S		T	T				<10	5.2
Q	S		T	I			2	<10	5.5
Q	S		T						5.8
Q	S		T	Y				<10	6.5
L	S			F			1	<20	10.8
L	S			V				<20	10.9
L	S			I			2	<20	11.3
L	S			Y				<20	11.3
L	S								14.5
L	S	S	T	L			1	<20	nd
L	S		T	T			1	<20	nd
L	S		T	Y			1	<15	nd
Q	S			Y			2	<10	nd
Q	S	S	T	L				<15	nd

Q	S		T	S				<20	nd
Q	S			T				<15	nd
	S		T					20	nd

Supplementary Table S5:

Steady-state kinetics parameters for the reduction of CB1954 by NADH catalysed by NfsB mutants.

Initial rates were monitored at 420 nm at a series of concentrations of both substrates and all the data fitted to the equation for a bi-bi substituted enzyme reaction (equation4), by nonlinear regression with equal weighting all points, using the programme Sigmaplot 14 (Systat software, San Jose, CA). The data for N71S/T41Q/F124T/M127V is from this work. The data for the other mutants is from [1]

Protein	k_{cat} s^{-1}	$K_{mCB1954}$ μM	$k_{cat}/K_{mCB1954}$ $\mu M^{-1}\ s^{-1}$	K_{mNADH} μM	k_{cat}/K_{mNADH} $\mu M^{-1}\ s^{-1}$
N71S/T41Q/F124T/M127V	270 ± 17	480 ± 60	0.57 ± 0.04	100 ± 13	2.7 ± 0.2
Wild type	140 ± 32	17200 ± 4800	0.007 ± 0.0006	40 ± 12	3.46 ± 0.6
T41L/N71S	153 ± 8	216 ± 33	0.71 ± 0.08	253 ± 41	0.62 ± 0.08
T41Q/N71S/F124T	181 ± 7	569 ± 45	0.32 ± 0.02	136 ± 12	1.33 ± 0.077

1 Jarrom, D., Jaberipour, M., Guise, C. P., Daff, S., White, S. A., Searle, P. F. and Hyde, E. I. (2009) Steady-state and stopped-flow kinetic studies of three Escherichia coli NfsB mutants with enhanced activity for the prodrug CB1954. Biochemistry. **48**, 7665-7672

Supplementary Table S6.

X-ray data collection and refinement statistics of T41Q/N71S/F124T/M127V NfsB bound to nicotinate.

PDB code	**8CJ0**
Data collection	
Wavelength (Å)	0.934
Resolution range (Å)	35.06-1.99 (2.05-1.99)
Space group	P 4_12_12
Unit cell	57.3 Å, 57.3 Å, 266 Å 90°, 90°, 90°
Total reflections	290,689 (19,436)
Unique reflections	31,129 (2,176)
Multiplicity	9.0 (8.93)
Completeness (%)	98.9 (95.3)
$\langle I/\sigma I \rangle$ overall	25.3 (6.2)
Mosaicity	0.141
Wilson B-factor (Å^2)	25.9

Anisotropy	0.167
Rsym[1] (%)	7.5 (55.0)
Refinement and quality	
Reflections used in refinement	31,128 (2168)
Reflections used for R-free	1,575 (5.1%)
R-work[2]	0.170
R-free[3]	0.205
Number of non-hydrogen atoms	3622
macromolecules	3344
ligands	84
solvent	155
Protein residues	
RMSD bond length (Å)	0.011
RMSD bond angles (°)	1.28
RMSD chirality	0.067
RMSD planarity	0.007

F_o-F_c correlation	0.96
Ramachandran plot	
Ramachandran favored (%)	99.5
Ramachandran allowed (%)	0.5
Ramachandran outliers (%)	0
Sidechain outliers (%)	1.7
All atom clash score	2
RSRZ outliers (%)	4.6
Average B-factors (Å^2)	34.0
macromolecules	36
ligands	
FMN	20
Nicotinamide	42
solvent	46.4

- The numbers in parentheses represent statistics in the highest resolution shell.

- *1.* $R_{sym} = \Sigma|I_i - \langle I \rangle|/\Sigma|I_i|$ where I_i is the intensity of the *i*th measurement, and $\langle I \rangle$ is the mean intensity for that reflection;
- *2.* $R_{work} = \sum||F_o| - |F_c|/\sum|F_o|$, where F_o and F_c are the observed and calculated structure factors for data used for refinement, respectively.
- *3.* $R_{free} = \sum||F_o| - |F_c|/\sum|F_o|$ for 5% of the data not used at any stage of structural refinement.