Electronic Supplementary Material (ESI) for Catalysis Science & Technology.

SUPPLEMENTARY MATERIAL

Catalytic mechanism of BsDyP an A-type dye-decolourising peroxidase: neither aspartate nor arginine is individually essential for peroxidase activity

Sónia Mendes[1], Teresa Catarino[1,2], Célia Silveira[1], Smilja Todorovic[1] and Lígia O. Martins[1]

[1]*Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República, 2780-157 Oeiras, Portugal*

[2]*Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal*

*Running title: *Role of distal residues in the catalysis of A-type BsDyP*

To whom correspondence should be addressed: Lígia O. Martins, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República, 2780-15 Oeiras, Portugal, E-mail: lmartins@itqb.unl.pt

Table S1. Relative populations of different coordination and spin states in WT BsDyP and variants, calculated from the relative contribution of each group in RR spectra, measured at pH 7.6, defined in component analysis of the experimental spectra.

	WT*	**D240N**	**R339L**	**D240N-R339L**	**N244L**	**D383N**
5cHS	0.22	Nd	/	/	/	/
6cHS	0.35	Nd	0.61	0.7	0.81	0.74
6cLS	0.43	Nd	0.39	0.3	0.19	0.26

* [44], Nd - not detected

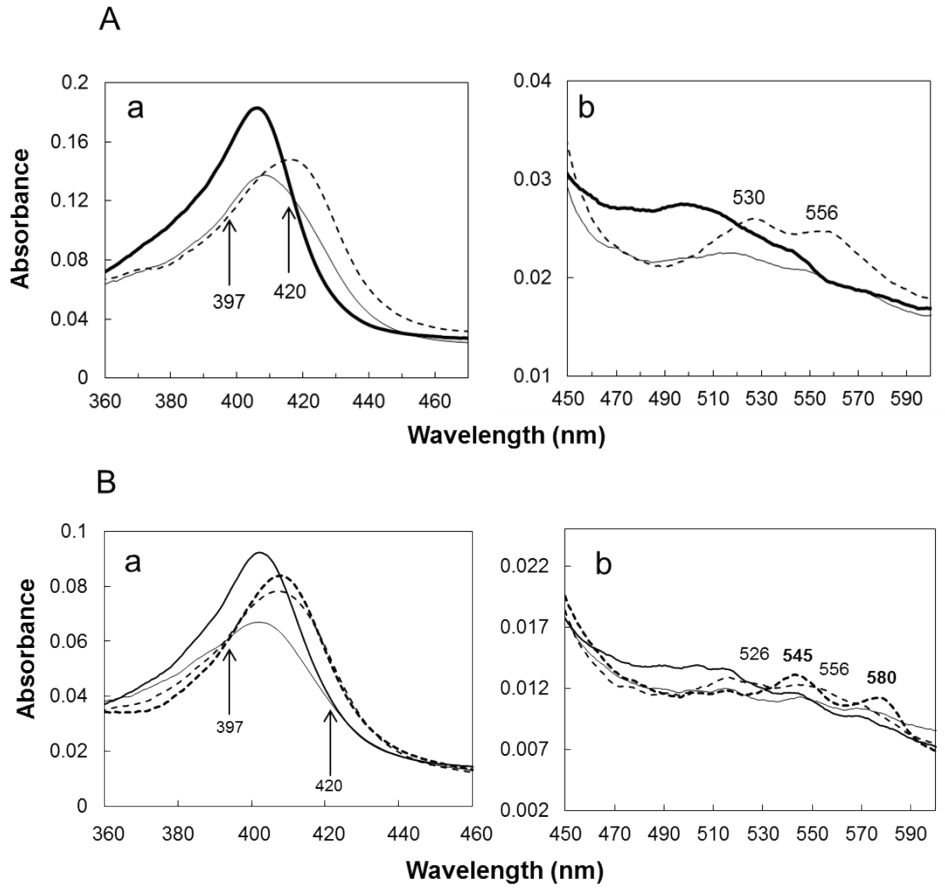

Figure S1 - Absorption spectra of resting BsDyP WT, Cpd I, Cpd II and Cpd III in the Soret region (a) and in the 450-600 nm region (b). The enzyme (~ 2 μM) was mixed with an equal volume of (**A**) 5 μM H_2O_2 or (**B**) 25 mM H_2O_2 in BR buffer, pH 7 at 25°C; the spectra recorded immediately after the mixing, correspond to the ferric state enzyme (*solid thick line*). Upon addition of low H_2O_2 concentrations to the resting enzyme **(A)** the spectra reveal the formation of one intermediate with the characteristics of Cpd I (*solid thin line*) prior to formation of Cpd II (*dashed thin line*). In the presence of a large excess of H_2O_2 **(B)** the resting enzyme decays to Cpd I, followed by Cpd II and Cpd III formation (*dashed thick line*). The arrows at 397 and 420 nm indicate the isosbestic point between Cpd I and Cpd II and between resting enzyme and Cpd I, respectively.

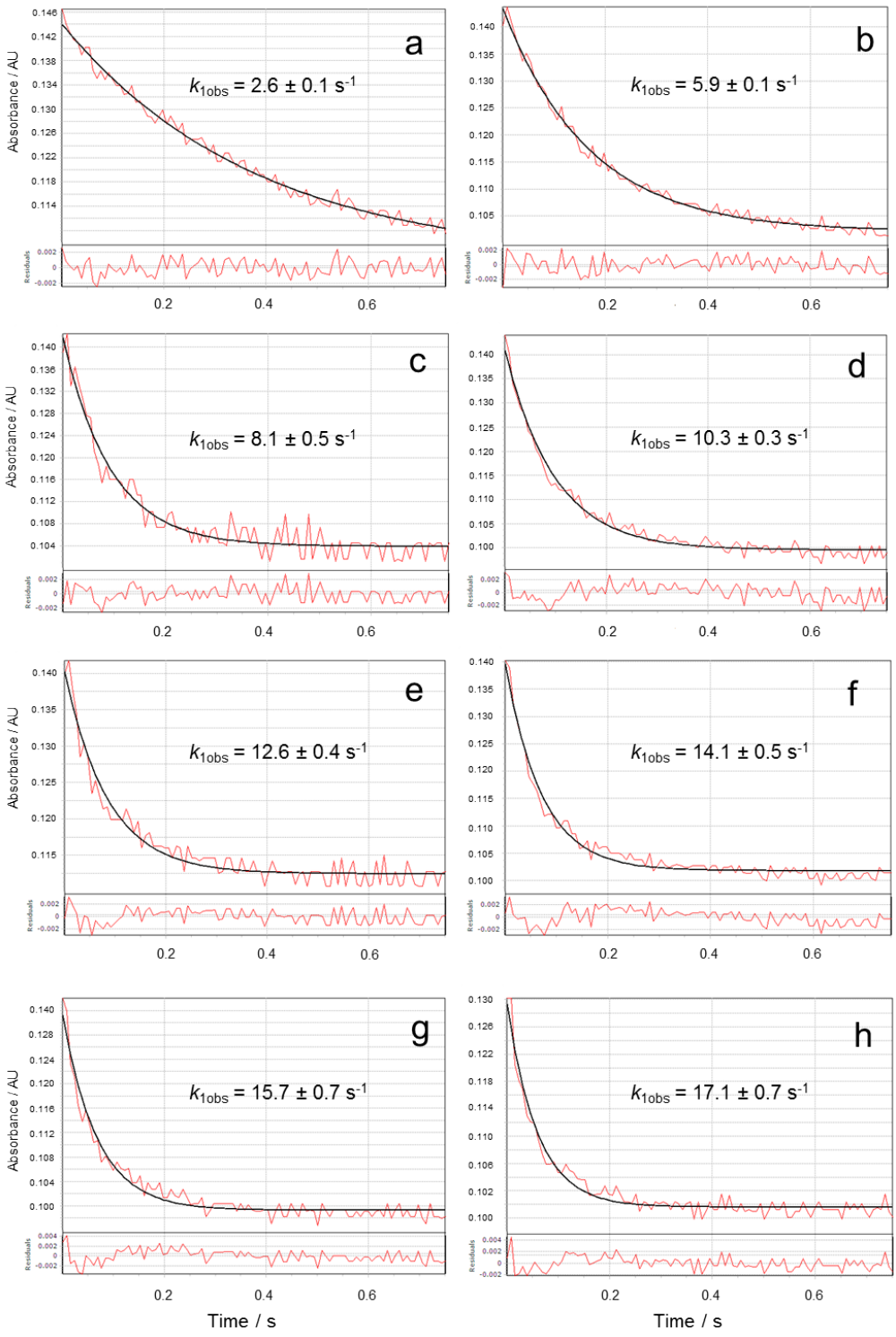

Figure S2 - Time course of BsDyP WT (~ 2 µM) the reaction with 2 µM (a), 5 µM (b), 7.5 µM (c), 10 µM (d), 15 µM (e), 20 µM (f), 30 µM $_2$ (g), and 50 µM H_2O_2 (h) followed at 397 nm. *Black* curves show the exponential fits to the representative data (in *red*). The rate constants indicated in each fit are the median of the values obtained from at least six independent traces.

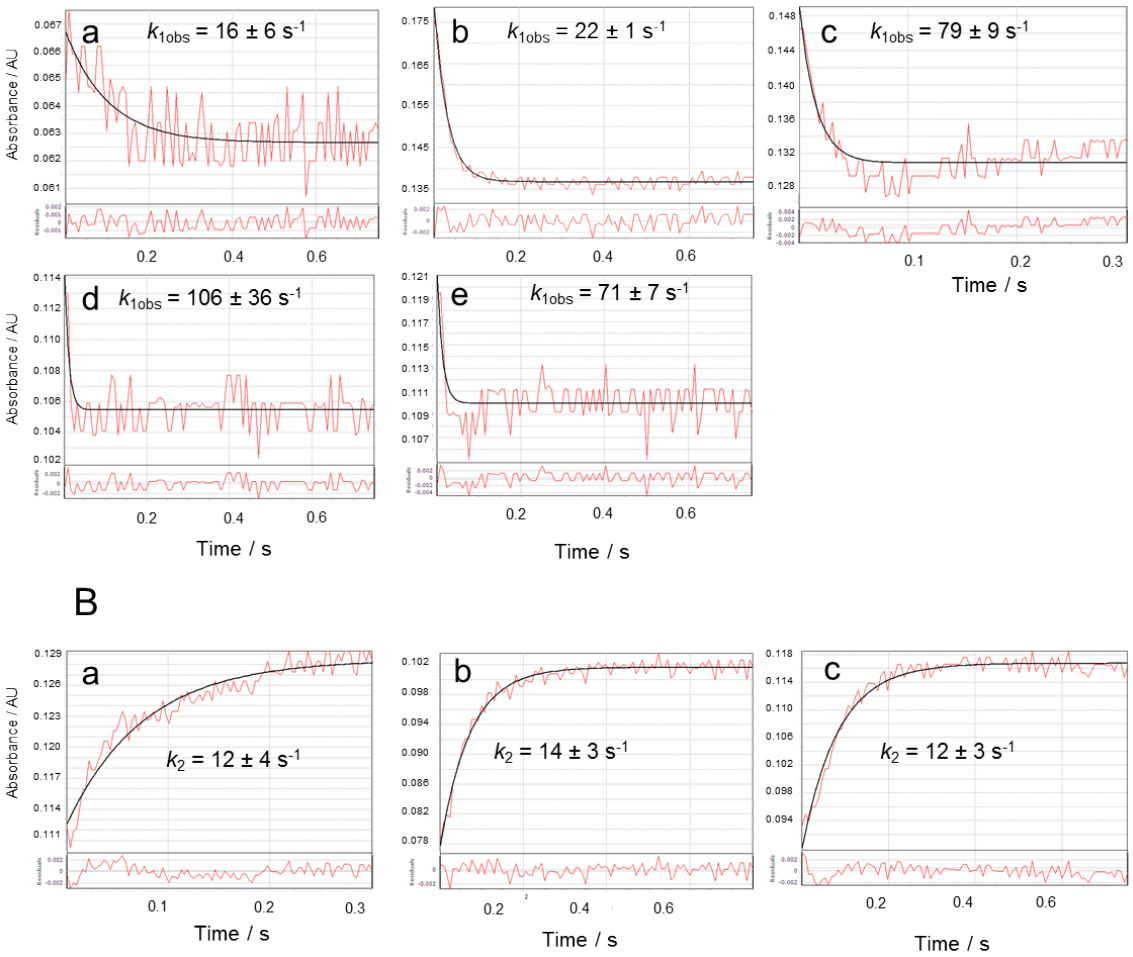

Figure S3 - Time course of BsDyP WT (~ 2 µM) reaction with 10 µM H_2O_2 followed at 397 nm (**A**) at pH 3 (a), pH 3.8 (b), pH 5 (c), pH 7 (d) and pH 9 (e) and followed at 420 nm (**B**) at pH 5 (a), pH 7 (b) and pH 9 (c). *Black* curves show the exponential fits to the representative data (in *red*). The rate constants indicated in each fit are the median of the values obtained from at least six independent traces.

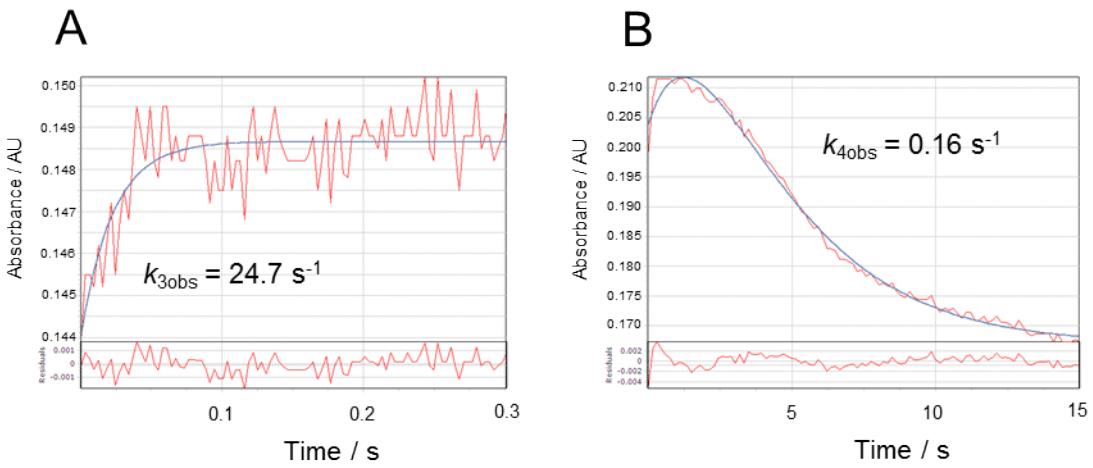

Figure S4 - Time course of the reaction of Cpd I and Cpd II with 0.1 mM guaiacol at pH 3.8 followed at 420 nm, the isosbestic point between Cpd I and native enzyme, where Cpd II formation (k_3) (**A**) and its subsequent conversion to the resting enzyme (k_4) can be measured (**B**). Blue curves show the exponential fits to the data (in *red*). The rate constants indicated in each fit are the median of the values obtained from at least six independent traces.

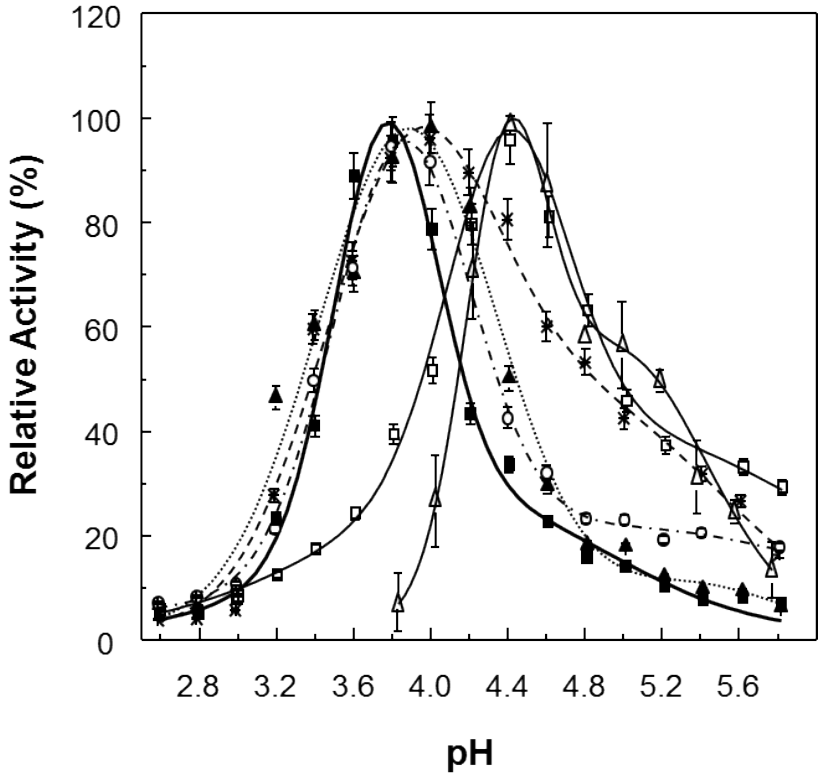

Figure S5 - pH activity profile for ABTS oxidation; WT (*black square*), D240N (*open square*), D383N (*open circle*), D240N-R339L (*open triangle*), R339L (*black triangle*) and N244L (*star*). The activity dependence on pH was measured by monitoring the oxidation of 1 mM (ABTS) in the presence of 0.2 mM H_2O_2 at 25°C using BR buffer (pH 2-7).

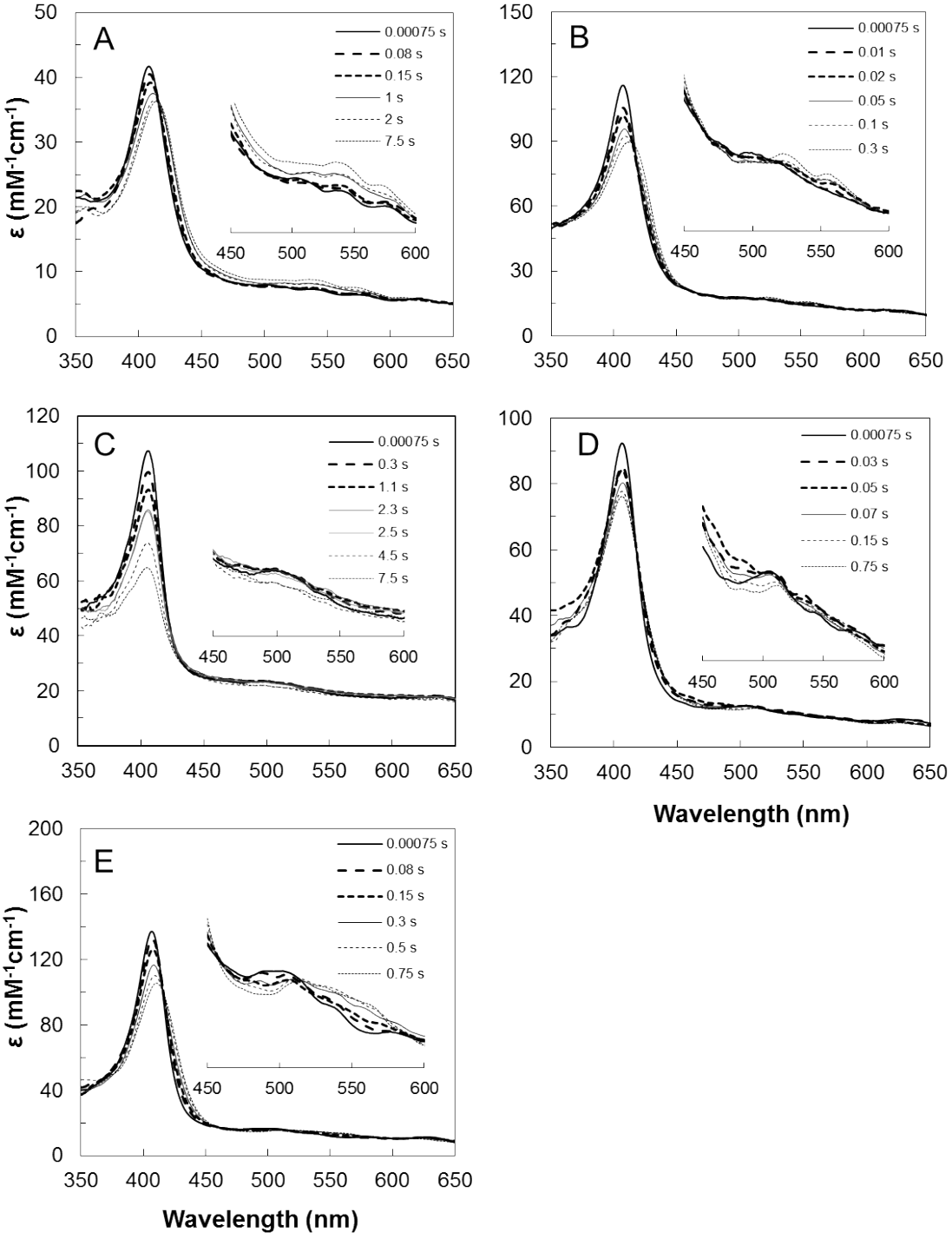

Figure S6 - Stopped-flow analysis of the reaction of D240N (A), R339L (B), D240N-R339L (C), N244L (D) and D383N (E) variants with H_2O_2 (BR buffer at pH 4.4 (A,C) and 3.8 (B,D,E) at 25°C). In each case, 2-4 μM enzyme was mixed with 200 μM H_2O_2 (A), 1000 μM H_2O_2 (B), 5000 μM H_2O_2 (C), 15 μM H_2O_2 (D) and 5 μM H_2O_2 (D). The spectra recorded immediately after the mixing, correspond to the ferric enzymes (*solid thick line*). The insets show the region between 450 and 600 nm.

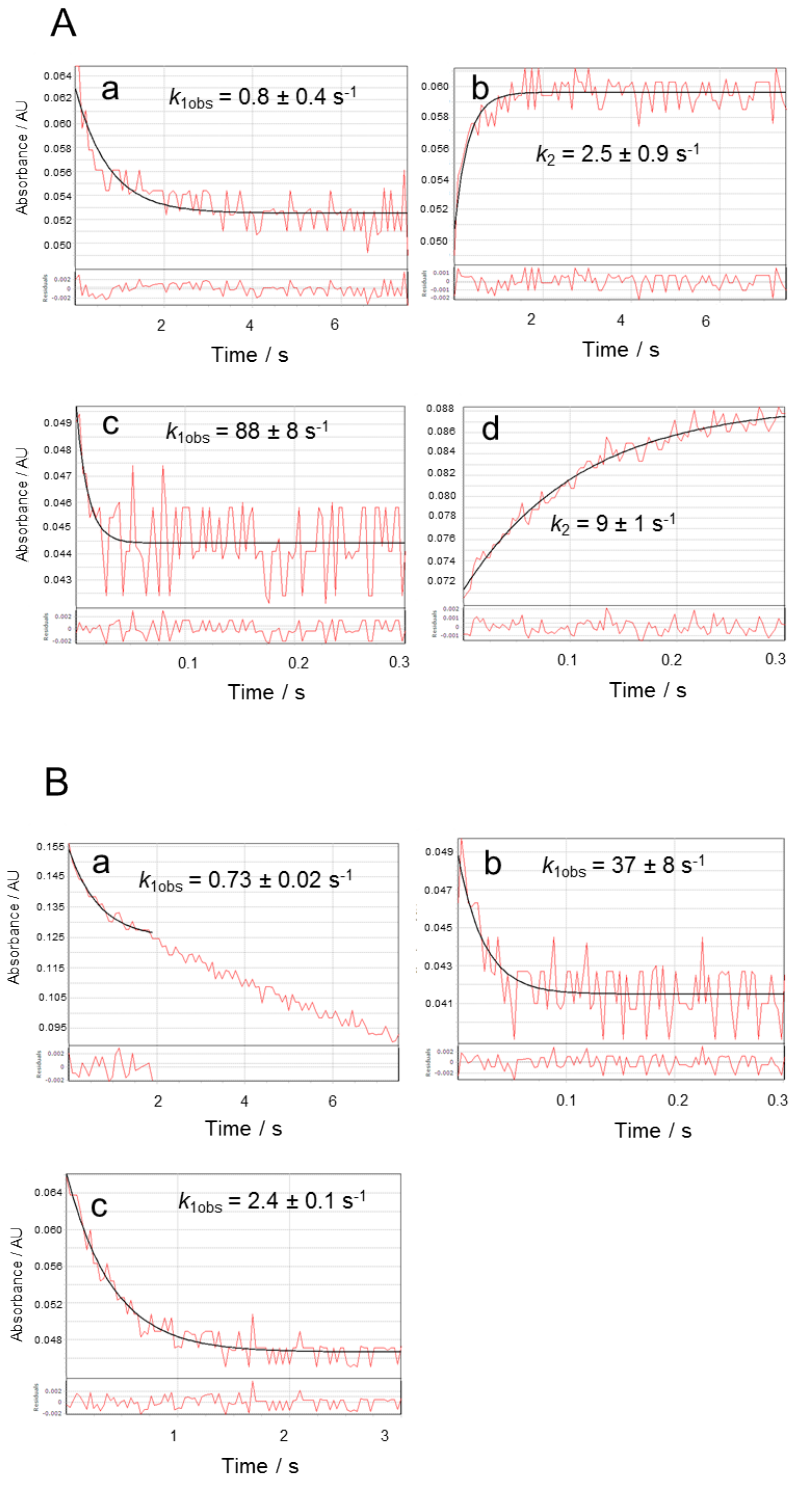

Figure S7- Time course of BsDyP variants reaction with H_2O_2 at the respective optimal pH for activity. (**A**) Reaction of D240N (a,b) with 200 µM H_2O_2 at pH 4.4 and of R339L (c,d) with 1000 µM H_2O_2 at pH 3.8 followed at 397 nm (a,c) and at 420 nm (b,d). (**B**) Reactions of D240N-R339L with 5000 µM H_2O_2 at pH 4.4 (a), of N244L with 15 µM H_2O_2 at pH 3.8 (b), and of D383N with 5 µM H_2O_2 at pH 3.8 (b) followed at 397 nm. *Black* curves show the exponential fits to the representative data (in *red*). The rate constants indicated in each fit are the median of the values obtained from at least six independent traces.

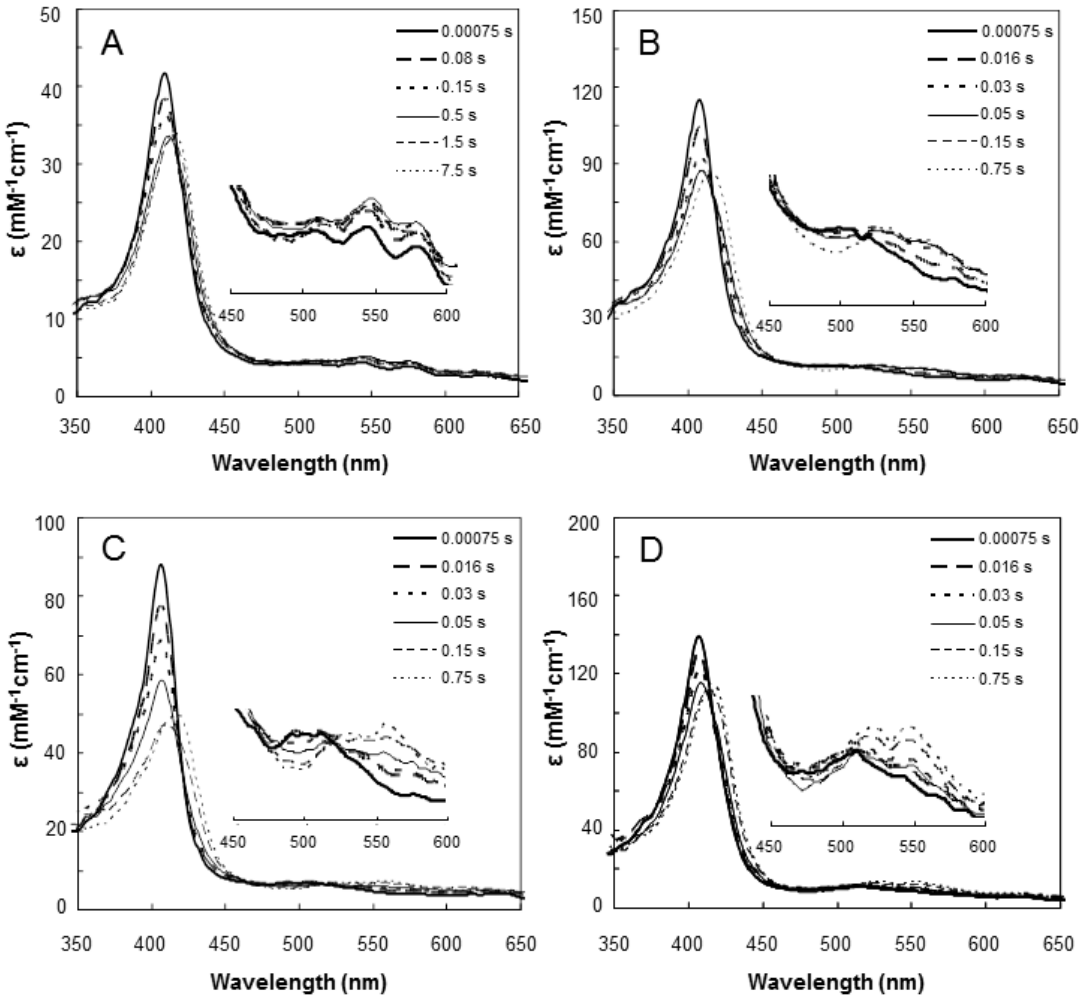

Figure S8- Stopped-flow analysis of the reaction of variants D240N (A), R339L (B), N244L (C) and D383N (D) with H_2O_2 (Britton-Robinson buffer, pH 7 at 25°C). In each case, 2 - 4 µM enzyme was mixed with 200 µM (A), 1000 µM (B), 15 µM (C) and 5 µM H_2O_2 (D). The spectra recorded immediately after the mixing correspond to the ferric enzymes (*solid thick line*). The spectral data reveal transitions of Cpd I (downshift of the Soret band) prior to formation of Cpd II (redshift of the Soret band). The insets show the region between 450 and 600 nm.

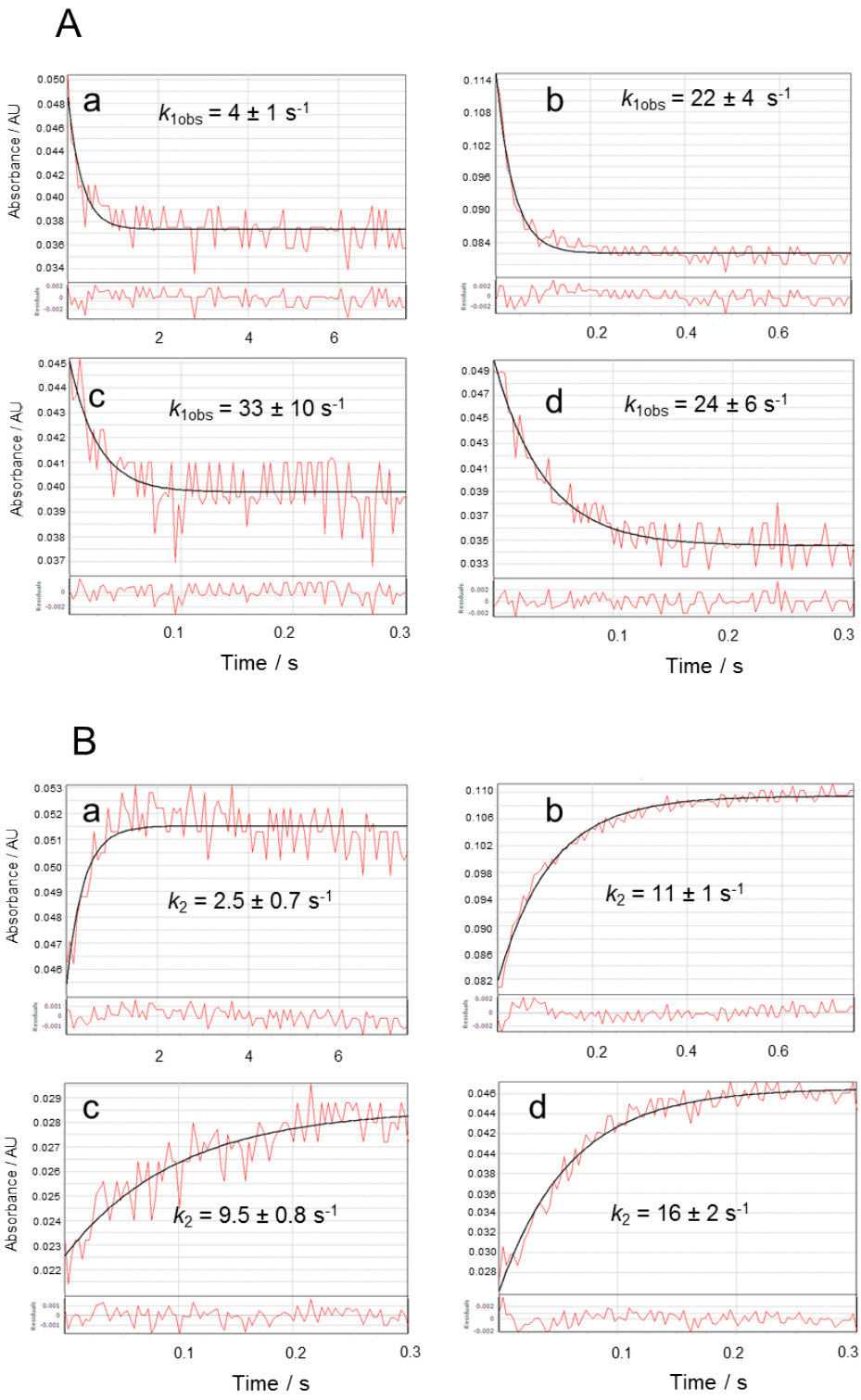

Figure S9 - Time course of BsDyP variants reaction with H_2O_2 at pH 7. The reactions were followed at 397 nm (**A**) and at 420 nm (**B**) after mixing D240N with 200 µM H_2O_2 (a), R339L with 1000 µM H_2O_2 (b), N244L with 15 µM H_2O_2 (c), and D383N with 5 µM H_2O_2 (d). *Black* curves show the exponential fits to the representative data (in *red*). The rate constants indicated in each fit are the median of the values obtained from at least six independent traces.

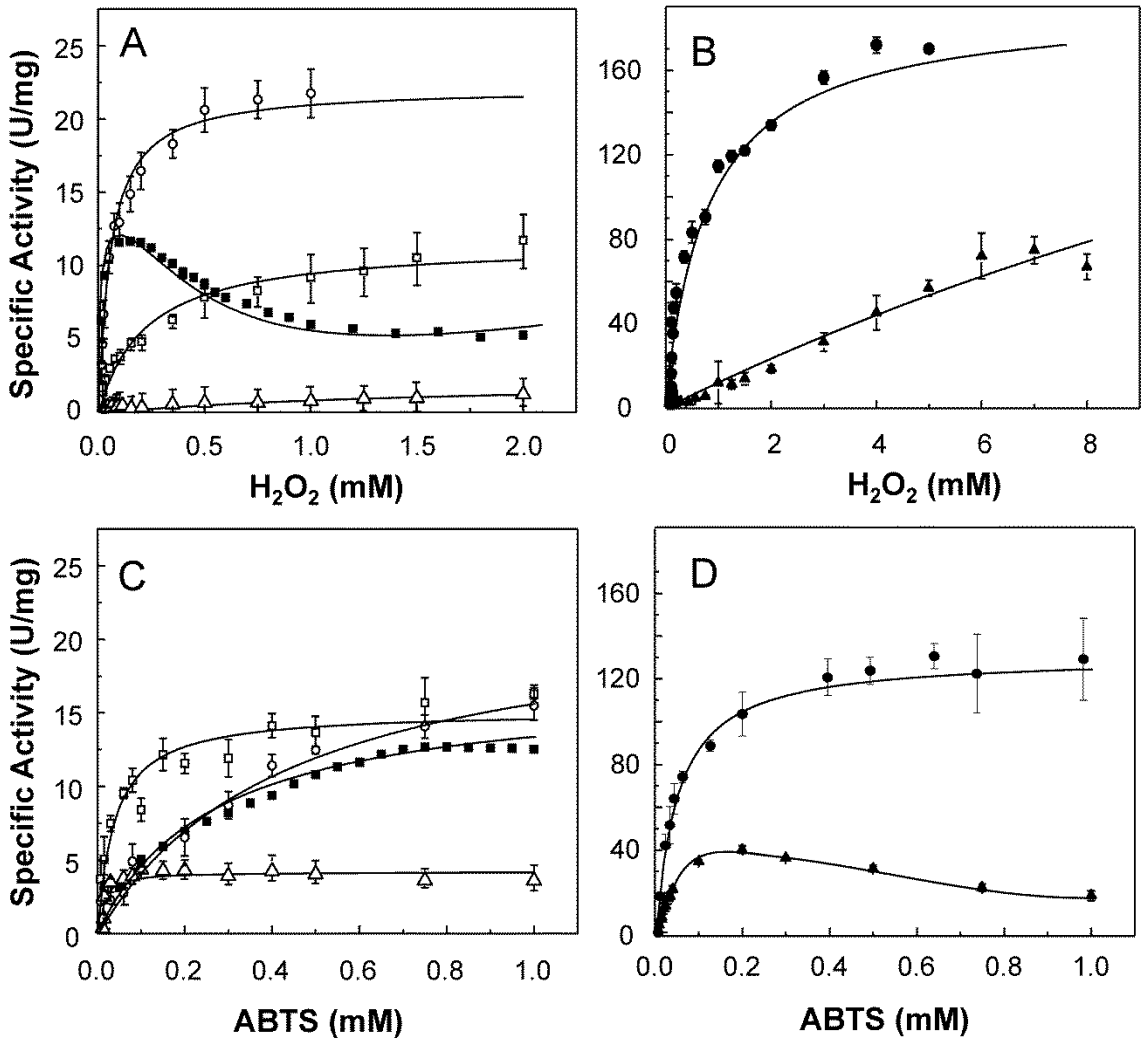

Fig. S10 - Apparent steady-state kinetics for H_2O_2 reduction (A, B) and ABTS oxidation (C, D); WT (*black square*), D240N (*open square*), D383N (*open circle*), D240N-R339L double mutant (*open triangle*), R339L (*black triangle*) and N244L (*black circle*) at the optimal pH for each enzyme. The kinetic parameters were fitted directly using the Michaelis-Menten equation or using the equation with substrate inhibition $\nu = V_{max}/(1+K_m/[S] + [S]/K_i)$ (for WT in A and R339L in D) (Origin-Lab software).