Electronic Supplementary Material (ESI) for Physical Chemistry Chemical Physics.

Supporting Information (Figs S1-S3, Tables S1-S7)

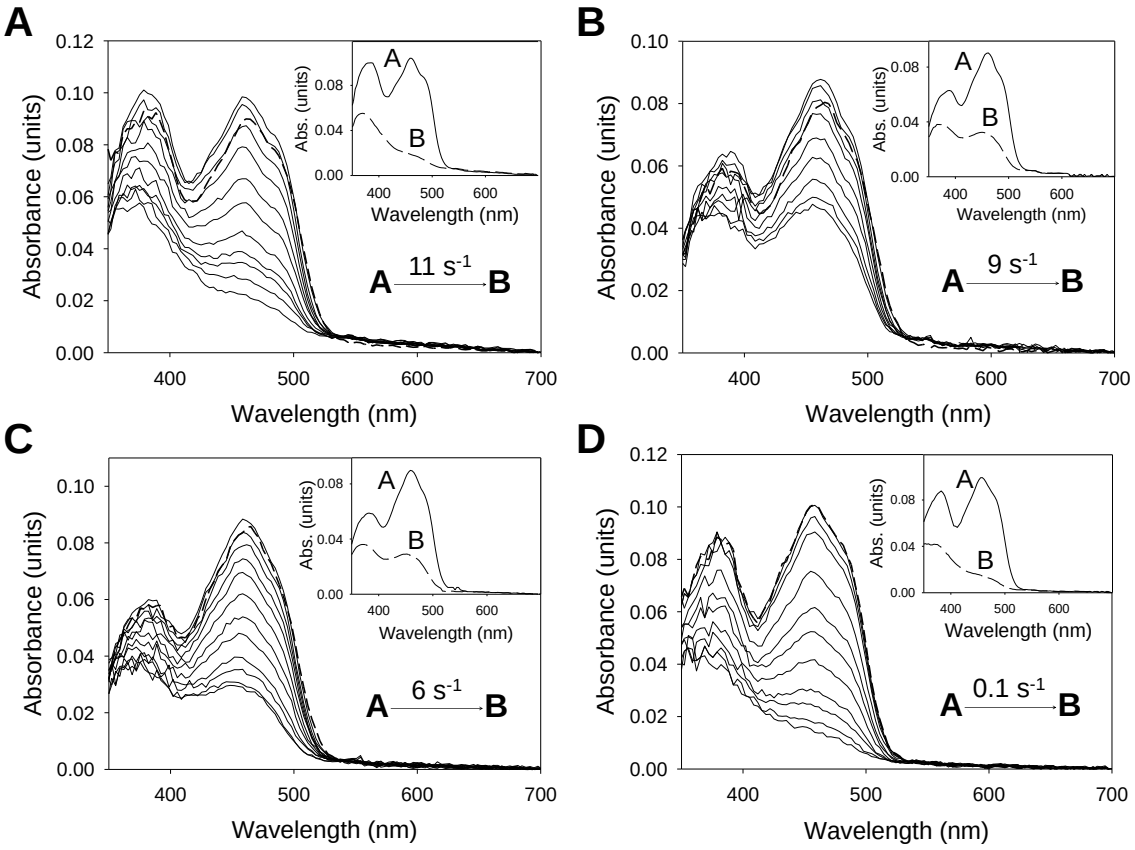

Fig. S1. Spectral evolution along the reduction of AAO variants by [α-$^2H_2$]-*p*-methoxybenzyl alcohol in 50 mM sodium phosphate, pH 6.0, at 12°C under anaerobic conditions. **A.** Native AAO (9 μM) with 31 μM of substrate at times: 0.004, 0.015, 0.02, 0.03, 0.05, 0.07, 0.1, 0.13, 0.15, 0.2 and 0.3 s. **B.** Y92F (9 μM) with 37 μM of substrate after: 0.001, 0.01, 0.02, 0.03, 0.05, 0.07, 0.1 0.13 and 0.15 s. **C.** Y92L (8 μM) with 37μM of substrate at times: 0.001, 0.02, 0.03, 0.05, 0.07, 0.1 0.15, 0.2, 0.3, 0.4, 0.6 and 0.8. **D.** Y92W (9 μM) with 312 μM of substrate after: 0.16, 0.5, 1, 3, 5, 7, 10, 15, 20, 30 and 60 s. Dashed lines represent the unbound enzyme spectra. Insets show spectral properties of initial (A) and final (B) species determined after global fitting of the reduction spectra.

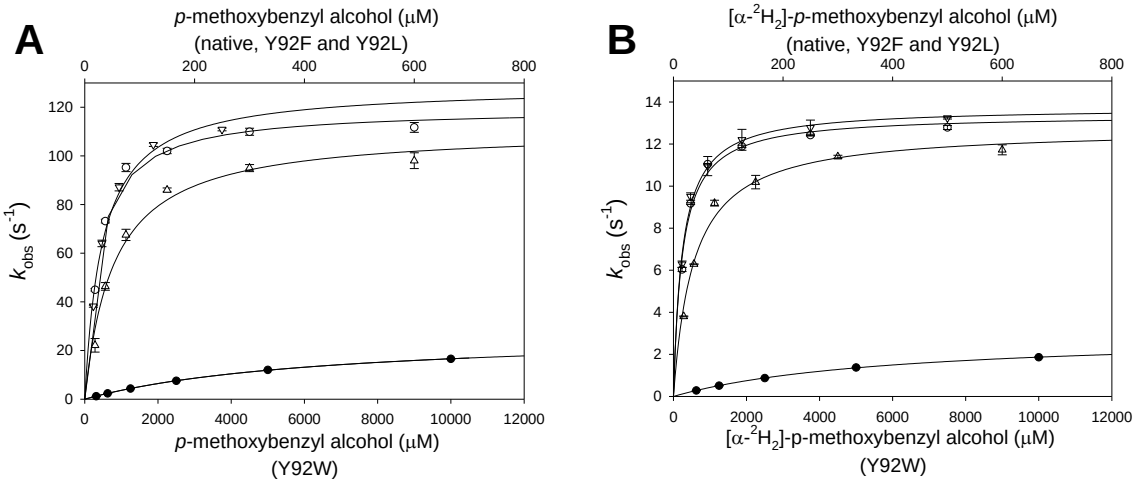

Fig. S2. k_{obs} for the reduction of native, Y92F and Y92L AAO variants (open circles, open inverted triangles and open triangles, respectively) at 14 °C (upper axis) and of Y92W variant (filled circles) at 25 °C (lower axis). **A.** Reduction by *p*-methoxybenzyl alcohol. **B.** Reduction by [α-$^2H_2$]-*p*-methoxybenzyl alcohol. Measurements by stopped-flow spectroscopy in 50 mM sodium phosphate, pH 6.0, under anaerobic conditions. Fits to equations describing hyperbolic behavior are shown. Data of the fits can be found in **Tables S3** and **S5-S7**.

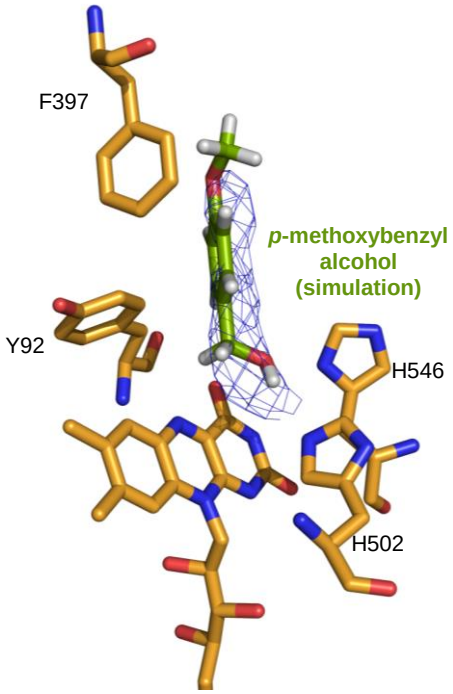

Fig. S3. Detail of the active site of the AAO:*p*-anisic acid complex (sticks in CPK, carbons in wheat) showing the electron density of the ligand in the crystal structure (PDB 5OC1) superimposed with the *p*-methoxybenzyl alcohol pose simulated by ligand migration with PELE[10] (sticks in CPK, carbons in pale green).

Table S1. X-ray data collection and refinement statistics for the AAO:*p*-anisic acid complex

Crystal data	**AAO:*p*-anisic acid complex**
Space group	$P6_422$
Unit cell parameters (Å)	a = b = 179.33, c = 160.18 α = β = 90°, γ = 120°
Data Collection	
Temperature (K)	100
Beamline	I24 DLS
Wavelength (Å)	0.96862
Resolution (Å)	43.99 - 2.30 (2.42 -2.30)
Total reflections	2710007 (392719)
Unique reflections	67615 (9716)
Mean I/σ(I)	28.1 (6.2)
Completeness (%)	100 (100)
Redundancy	40.1 (40.4)
R_{merge}[a]	0.133 (0.806)
Data Refinement	
Resolution range (Å)	42.3 -2.30
Protein non-hydrogen atoms	4299
Ligand non-hydrogen atoms	94
Solvent non-hydrogen atoms	280
R_{work} (%)	17.43
R_{free}[b] (%)	19.50
r.m.s.d. bond length (Å)	0.012
r.m.s.d. bond angles (°)	1.661
Average B-factor (Å^2)	32.93

Values in parentheses correspond to the highest resolution shell.
[a]$R_{merge} = \Sigma|I - I_{av}| / \Sigma I$, where the summation is over symmetry-equivalent reflection.
[b]R calculated for 7% of data excluded from the refinement.

Table S2. Estimated k_{cat}, K_m and k_{cat}/K_m steady-state parameters of native AAO with protiated and di-deuterated *p*-methoxybenzyl alcohols at different temperatures

T (°C)	***p*-methoxybenzyl alcohol**			**[α-$^2H_2$]-*p*-methoxybenzyl alcohol**		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1}\cdot\mu M^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1}\cdot\mu M^{-1}$)
10	118 ± 2	32.0 ± 1.7	3.0 ± 0.2	7.6 ± 0.3	8.8 ± 1.1	0.86 ± 0.12
15	157.2 ± 4.3	54.9 ± 3.7	3.5 ± 0.3	9.0 ± 0.2	12.4 ± 1.1	0.73 ± 0.07
20	177.3 ± 2.3	42.8 ± 1.4	4.1 ± 0.1	19.4 ± 0.2	20.1 ± 0.6	0.96 ± 0.17
25[a]	197.0 ± 2.0	49.0 ± 1.0	4.0 ± 0.3	25.0 ± 0.2	24.6 ± 2.5	1.02 ± 0.13
30	268.4 ± 15.5	53.9 ± 7.3	5.0 ± 0.7	49.6 ± 1.3	48.6 ± 3.2	1.02 ± 0.07

Bi-substrate kinetics were performed with α-protiated and [α-$^2H_2$]-*p*-methoxybenzyl alcohols in 50 mM sodium phosphate pH 6. [a]Data from Hernández-Ortega *et al.* (2012). Means ± S.D.

Table S3. Reduction (k_{red}) and dissociation (K_d) constants for WT AAO with protiated, mono- and di-deuterated *p*-methoxybenzyl alcohols at different temperatures

T (°C)	***p*-methoxybenzyl alcohol**		**[(*R*)-^2H]-*p*-methoxybenzyl alcohol**		**[α-$^2H_2$]-*p*-methoxybenzyl alcohol**	
	k_{red} (s^{-1})	K_d (μM)	k_{red} (s^{-1})	K_d (μM)	k_{red} (s^{-1})	K_d (μM)
6	75.6 ± 2.4	15.7 ± 2.8	10.7 ± 0.1	15.8 ± 0.4	7.7 ± 0.1	8.4 ± 0.8
8	81.1 ± 0.8	21.4 ± 0.5	11.7 ± 0.1	18.7 ± 0.5	8.3 ± 0.1	6.7 ± 0.6
10	89.0 ± 2.0	22.6 ± 1.4	14.7 ± 0.1	18.0 ± 0.4	9.7 ± 0.1	9.3 ± 0.6
12	95.9 ± 1.8	22.0 ± 1.2	17.8 ± 0.2	20.0 ± 1.2	11.1 ± 0.1	10.0 ± 0.2
14	112.0 ± 1.6	24.4 ± 1.0	20.8 ± 0.9	25.7 ± 4.9	12.7 ± 0.1	16.6 ± 0.5

Measurements were carried out in 50 mM sodium phosphate buffer pH 6. Means ± S.D.

Table S4. Secondary KIE on the reduction rate (k_{red}) of native AAO with α-deuterated *p*-methoxybenzyl alcohols

T (°C)	KIE
6	1.39 ± 0.02
8	1.41 ± 0.02
10	1.52 ± 0.02
12	1.60 ± 0.02
14	1.63 ± 0.08

k_{red} were calculated with (*R*)-[α-^2H] and [α-$^2H_2$]-*p*-methoxybenzyl alcohols. Secondary KIEs were estimated as the ratio between HT constants of monodeuterated and di-deuterated substrates. Value ± S.D.

Table S5. Reduction (k_{red}) and dissociation (K_d) constants for Y92F AAO with protiated and di-deuterated *p*-methoxybenzyl alcohol

T (°C)	***p*-methoxybenzyl alcohol**		**[α-$^2H_2$]-*p*-methoxybenzyl alcohol**	
	k_{red} (s^{-1})	K_d (μM)	k_{red} (s^{-1})	K_d (μM)
6	83.4 ± 2.9	22.7 ± 2.1	8.3 ± 0.1	12.9 ± 0.7
8	89.1 ± 1.7	22.0 ± 1.1	9.2 ± 0.1	13.2 ± 0.6
10	102.7 ± 1.0	25.3 ± 0.6	10.7 ± 0.2	15.7 ± 1.0
12	108.4 ± 0.8	29.6 ± 0.6	12.3 ± 0.1	17.0 ± 0.4
14	117.6 ± 1.8	27.3 ± 0.9	13.3 ± 0.3	16.4 ± 1.0

Measurements were carried out in 50 mM sodium phosphate buffer pH 6. Means ± S.D.

Table S6. Reduction (k_{red}) and dissociation (K_d) constants for Y92L AAO with protiated and di-deuterated *p*-methoxybenzyl alcohol

T (°C)	***p*-methoxybenzyl alcohol**		**[α-$^2H_2$]-*p*-methoxybenzyl alcohol**	
	k_{red} (s^{-1})	K_d (μM)	k_{red} (s^{-1})	K_d (μM)

6	72.3 ± 2.8	36.8 ± 3.8	7.3 ± 0.1	25.2 ± 1.6
8	76.4 ± 0.7	42.2 ± 0.9	8.3 ± 0.1	31.4 ± 0.6
10	82.4 ± 0.9	45.0 ± 1.2	9.6 ± 0.2	29.6 ± 1.3
12	90.1 ± 0.4	47.8 ± 0.7	11.0 ± 0.2	31.1 ± 1.6
14	100.6 ± 1.4	43.7 ± 1.5	12.7 ± 0.3	33.4 ± 2.0

Measurements were carried out in 50 mM sodium phosphate buffer pH 6. Means ± S.D.

Table S7. Reduction (k_{red}) and dissociation (K_d) constants for Y92W AAO with protiated and di-deuterated *p*-methoxybenzyl alcohol

T (°C)	***p*-methoxybenzyl alcohol**		**[α-$^2H_2$]-*p*-methoxybenzyl alcohol**	
	k_{red} (s^{-1})	K_d (mM)	k_{red} (s^{-1})	K_d (mM)
12	13.9 ± 0.8	4.6 ± 0.5	1.5 ± 0.1	4.3 ± 0.2
16	17.5 ± 0.1	3.8 ± 0.1	1.8 ± 0.2	4.3 ± 0.9
20	22.8 ± 0.5	5.3 ± 0.4	2.3 ± 0.2	5.0 ± 0.6
25	27.6 ± 0.3	6.6 ± 0.1	3.0 ± 0.1	6.0 ± 0.2

Measurements were carried out in 50 mM sodium phosphate buffer pH 6. Means ± S.D.