Structural dynamics of cytochrome P450 3A4 in the presence of substrates and cytochrome P450 reductase

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SUPPORTING INFORMATION

Data Set	CYP3A4	CYP3A4 + rCPR*ox*	CYP3A4 + rCPR*red*
HDX reaction details	0.1 M KPi, pD = 7.0, RT, 1 μM CYP	0.1 M KPi, pD = 7.0, RT, 1 μM CYP, 4μM CPR	0.1 M KPi, pD = 7.0, RT, 1 μM CYP, 4μM CPR, 1mM NADPH
HDX time course (min)	0.5, 5, 30, 60	0.5, 5, 30	0.5, 5, 30
# of Peptides	65	65	64
Sequence coverage (%)	78.9	81.1	76.5
Redundancy	1.94	1.87	1.91
Replicates	3 technical	3 technical	3 technical
Repeatability (maximum SD between replicates, Da)	0.39	0.22	0.52

Data Set	CYP3A4 + BFC	CYP3A4 + TST	CYP3A4 + PRG
HDX reaction details	0.1 M KPi, pD = 7.0, RT, 1 μM CYP, 100 μM BFC	0.1 M KPi, pD = 7.0, RT, 1 μM CYP, 100 μM TST	0.1 M KPi, pD = 7.0, RT, 1 μM CYP, 100 μM PRG
HDX time course (min)	5, 60	5, 60	5, 60
# of Peptides	58	62	56
Sequence coverage (%)	76.3	78.9	74.8
Redundancy	1.83	1.89	1.77
Replicates	3 technical	3 technical	3 technical
Repeatability (maximum SD between replicates, Da)	0.62	0.58	0.58

Table S1. Summary tables of HDX-MS results for all experimental conditions tested. All experiments were performed under the same conditions, but the number of D_2O exposure time points varied. The number and nature of the detected peptides varied slightly, but the percent sequence coverage remained higher than 75% for all states. Redundancy is a measure of the average number of peptides that cover each amino acid.

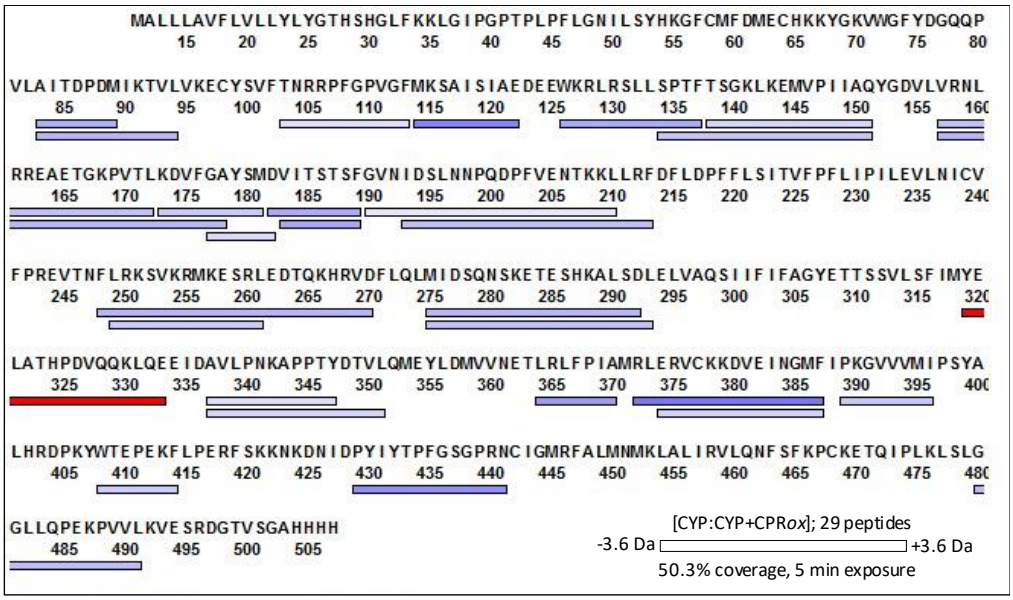
Human CYP3A4 (Δ3-13)

MALLLAVFLVLLYLYGTHSHGLFKKLGIPGPTPLPFLGNILSYHKGFCMFDMECHKKYGKVWGFYDGQQPVLAITDPDMIKTVLVKECYSVFTNR
RPFGPVGFMKSAISIAEDEEWKRLRSLLSPTFTSGKLKEMVPIIAQYGDVLVRNLRREAETGKPVTLKDVFGAYSMDVITSTSFGVNIDSLNNPQ
DPFVENTKKLLRFDFLDPFFLSITVFPFLIPILEVLNICVFPREVTNFLRKSVKRMKESRLEDTQKHRVDFLQLMIDSQNSKETESHKALSDLELVA
QSIIFIFAGYETTSSVLSFIMYELATHPDVQQKLQEEIDAVLPNKAPPTYDTVLQMEYLDMVVNETLRLFPIAMRLERVCKKDVEINGMFIPKGVVV
MIPSYALHRDPKYWTEPEKFLPERFSKKNKDNIDPYIYTPFGSGPRNCIGMRFALMNMKLALIRVLQNFSFKPCKETQIPLKLSLGGLLQPEKPVV
LKVESRDGTVSGAHHHH

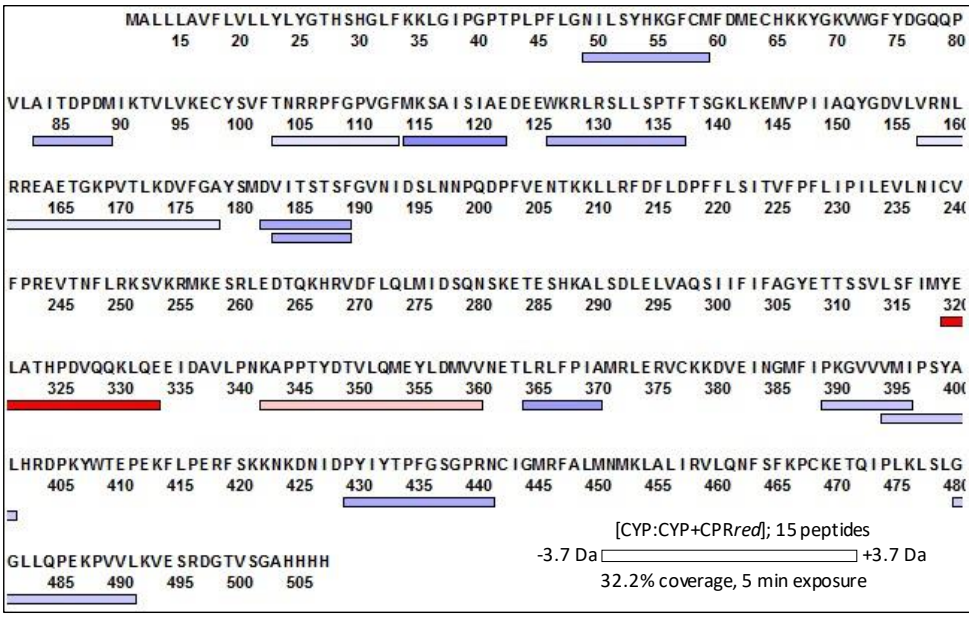
Rat CPR

MGDSHEDTSATMPEAVAEEVSLFSTTDMVLFSLIVGVLTYWFIFRKKKEEIPEFSKIQTTAPPVKESSFVEKMKKTGRNIIVFYGSQTGTAEEFAN
RLSKDAHRYGMRGMSADPEEYDLADLSSLPEIDKSLVVFCMATYGEGDPTDNAQDFYDWLQETDVDLTGVKFAVFGLGNKTYEHFNAMGKYV
DQRLEQLGAQRIFELGLGDDDGNLEEDFITWREQFWPAVCEFFGVEATGEESSIRQYELVVHEDMDVAKVYTGEMGRLKSYENQKPPFDAKN
PFLAAVTANRKLNQGTERHLMHLELDISDSKIRYESGDHVAVYPANDSALVNQIGEILGADLDVIMSLNNLDEESNKKHPFPCPTTYRTALTYYLD
ITNPPRTNVLYELAQYASEPSEQEHLHKMASSSGEGKELYLSWVVEARRHILAILQDYPSLRPPIDHLCELLPRLQARYYSIASSSKVHPNSVHIC
AVAVEYEAKSGRVNKGVATSWLRAKEPAGENGGRALVPMFVRKSQFRLPFKSTTPVIMVGPGTGIAPFMGFIQERAWLREQGKEVGETLLYY
GCRRSDEDYLYREELARFHKDGALTQLNVAFSREQAHKVYVQHLLKRDREHLWKLIHEGGAHIYVCGDARNMAKDVQNTFYDIVAEFGPMEH
TQAVDYVKKLMTKGRYSLDVWS

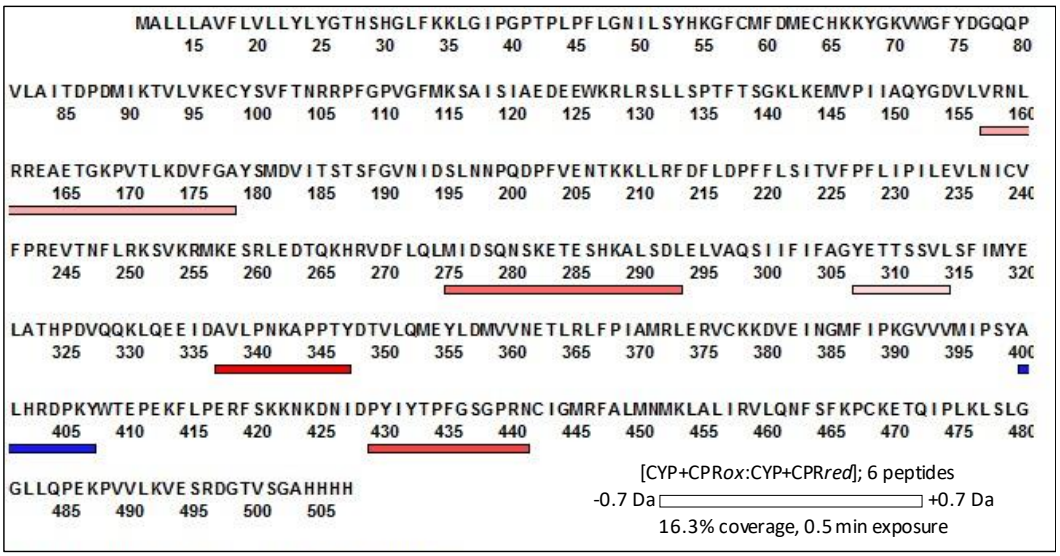
Figure S1. Amino acid sequence of human CYP3A4 and rat CPR.



Deuterium uptake difference: [CYP:CYP+CPR*ox*]			
Start	**End**	**Sum Da**	**Sum RFU**
83	89	0.82	16.75
83	94	1.42	14.51
103	113	0.69	8.79
114	122	5.45	69.50
126	137	3.25	33.17
134	151	0.79	5.35
138	151	0.61	5.20
157	172	2.55	18.62
157	178	1.20	6.10
173	181	0.84	10.65
177	182	0.74	15.06
182	189	1.23	17.89
183	189	3.39	57.69
190	210	0.86	4.89
193	213	2.35	13.29
248	270	1.18	5.48
249	261	0.66	5.64
275	292	1.23	7.39
275	293	2.16	12.25
319	333	-7.78	-60.65
337	347	1.10	16.08
337	351	0.61	5.64
364	370	3.00	61.32
372	387	3.41	23.21
374	387	2.08	16.33
389	396	0.69	11.70
408	414	0.72	14.68
429	441	2.25	25.52
480	491	1.89	21.43

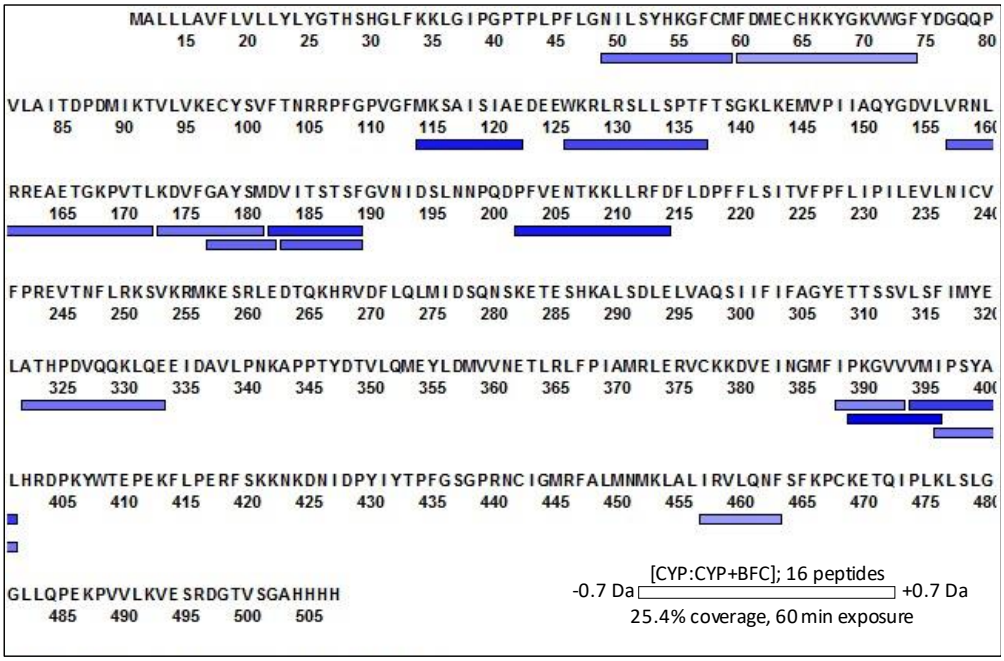


Deuterium uptake difference [CYP:CYP+CPR*red*]			
Start	**End**	**Sum Da**	**Sum %RFU**
49	59	1.52	15.48
83	89	0.76	15.58
103	113	0.75	9.51
114	122	5.26	67.05
126	137	2.11	21.55
157	178	0.98	4.98
182	189	2.50	36.51
183	189	1.00	16.94
319	333	-7.88	-61.84
342	360	-0.77	-4.94
364	370	4.19	85.57
389	396	2.41	40.95
394	401	0.82	13.93
429	441	2.95	33.45
480	491	1.05	11.87

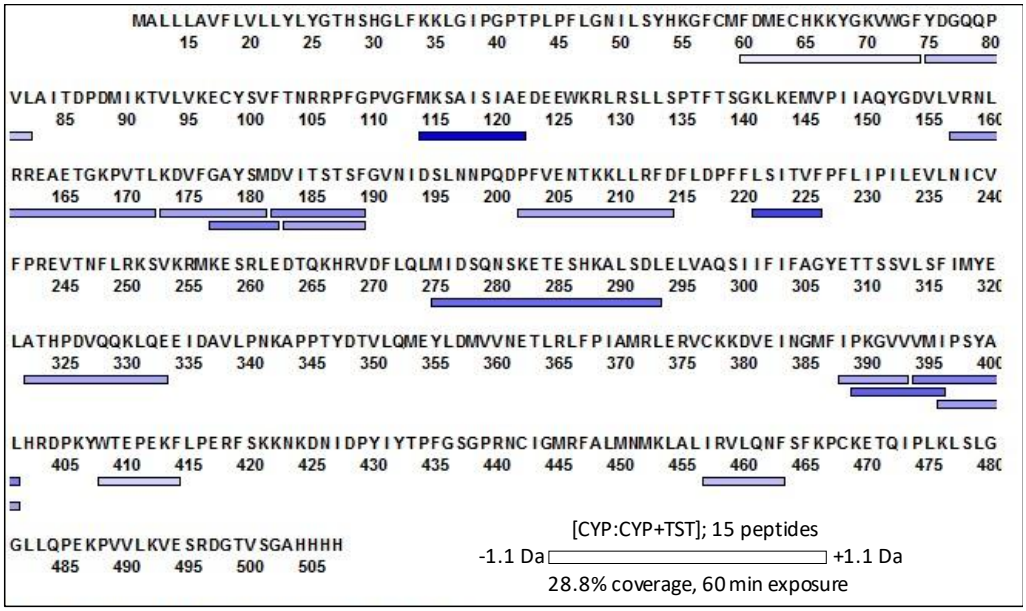


Deuterium uptake difference [CYP+CPR*ox*:CYP+CPR*red*]			
Start	**End**	**Sum Da**	**Sum RFU**
157	178	0.68	3.49
275	293	0.64	3.63
307	314	0.90	13.12
337	347	0.65	9.46
400	407	-0.65	-11.00
429	441	0.50	5.62

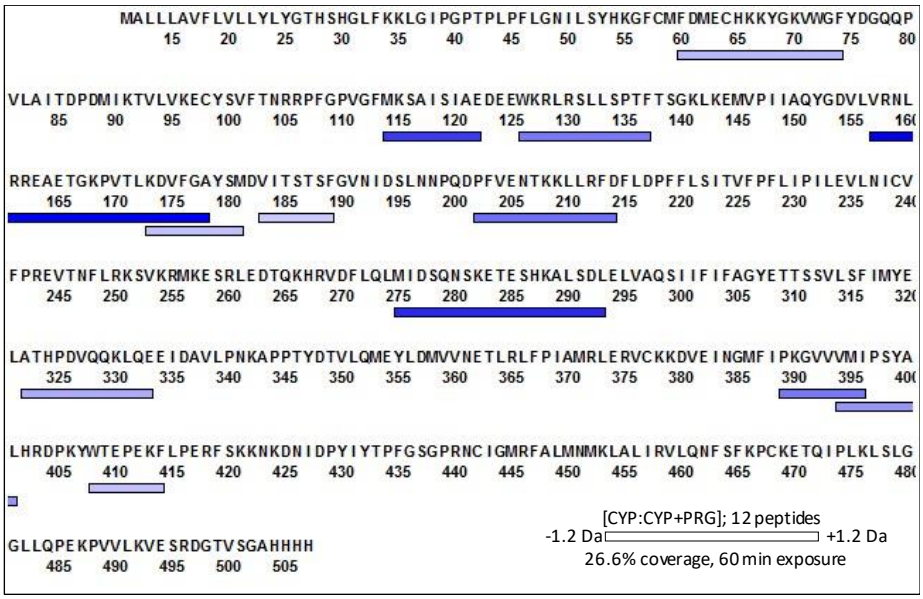
Figure S2. Coverage map and tables of CYP3A4-derived peptides that undergo a significant change in deuterium uptake in the presence of *ox*CPR or *red*CPR ([CYP] – [CYP:CPR*ox*] CI 99.9%: ± 0.60 Da; [CYP] – [CYP+CPR*red*] CI 99.9%: 0.69 ± Da; [CYP:CPR*ox*] – [CYP:CPR*red*] CI 99%: ± 0.34 Da). Coverage map bars and table entries shown in blue represent peptides that become more rigid in the presence of *ox*CPR or *red*CPR (i.e. less deuterium uptake), whereas peptides shaded in red become more flexible under the same conditions (i.e. more deuterium uptake).



Deuterium uptake difference: [CYP:CYP+BFC]			
Start	**End**	**Sum Da**	**Sum %RFU**
49	59	0.40	4.07
60	74	0.40	2.94
114	122	1.67	21.34
126	137	0.65	6.60
157	172	0.36	2.63
173	181	0.42	5.38
177	182	0.41	8.47
182	189	0.84	12.21
183	189	0.82	13.97
202	214	0.56	5.19
322	333	0.37	3.76
388	393	0.38	9.63
389	396	0.68	11.59
394	401	0.66	11.22
396	401	0.78	20.02
457	463	0.39	6.60



Deuterium uptake difference: [CYP:CYP+TST]			
Start	**End**	**Sum Da**	**Sum %RFU**
60	74	0.52	3.83
75	82	0.41	7.05
114	122	1.14	14.61
157	172	0.67	4.86
173	181	0.60	7.71
177	182	0.42	8.62
182	189	0.91	13.29
183	189	0.86	14.66
202	214	1.04	9.67
221	226	0.76	15.54
275	293	0.63	3.55
322	333	0,53	5.40
388	393	0.76	19.50
389	396	1.37	23.30
394	401	0.68	11.64
396	401	0.41	10.39
408	414	0.41	8.35
457	463	0.42	7.09



Deuterium uptake difference: [CYP:CYP+PRG]			
Start	**End**	**Sum Da**	**Sum %RFU**
60	74	0.48	3.48
114	122	0.92	11.69
126	137	0.63	6.38
157	178	1.15	5.87
173	181	0.50	6.37
183	189	0.66	11.15
202	214	0.88	8.13
275	293	0.72	4.09
322	333	0.48	4.92
389	396	1.17	19.97
394	401	0.63	10.64
408	414	0.49	9.93

Figure S3. Coverage map and tables of CYP3A4-derived peptides that undergo a significant change in deuterium in the presence of either BFC, testosterone (TST), or progesterone (PRG) ([CYP] – [CYP:BFC] CI 99%: ± 0.34 Da; [CYP] – [CYP:TST] CI 99%: 0.36 ± Da; [CYP] – [CYP+PRG] CI 99%: ± 0.45 Da). Coverage map bars and table entries highlighted in blue represent peptides becoming more rigid in the presence of substrates (i.e. less deuterium uptake).

Figure S4. Butterfly plots mapping the significant differences among peptides: **A**) [CYP] – [CYP:*ox*CPR] (CI 99.9%); **B**) [CYP] – [CYP:*red*CPR] (CI 99.9%); **C**) [CYP:*ox*CPR] – [CYP:*red*CPR] (CI 99%); **D**) [CYP] – [CYP:BFC]; **E**) [CYP] – [CYP:TST]; **F**) [CYP] – [CYP:PRG]. Each dot represents a peptide and, when the dot is colored, it becomes considerably more rigid or more flexible in the presence of added CPR or substrate. For each set, the color coding is explained on the right. The y axis represents the uptake difference measured in Da.

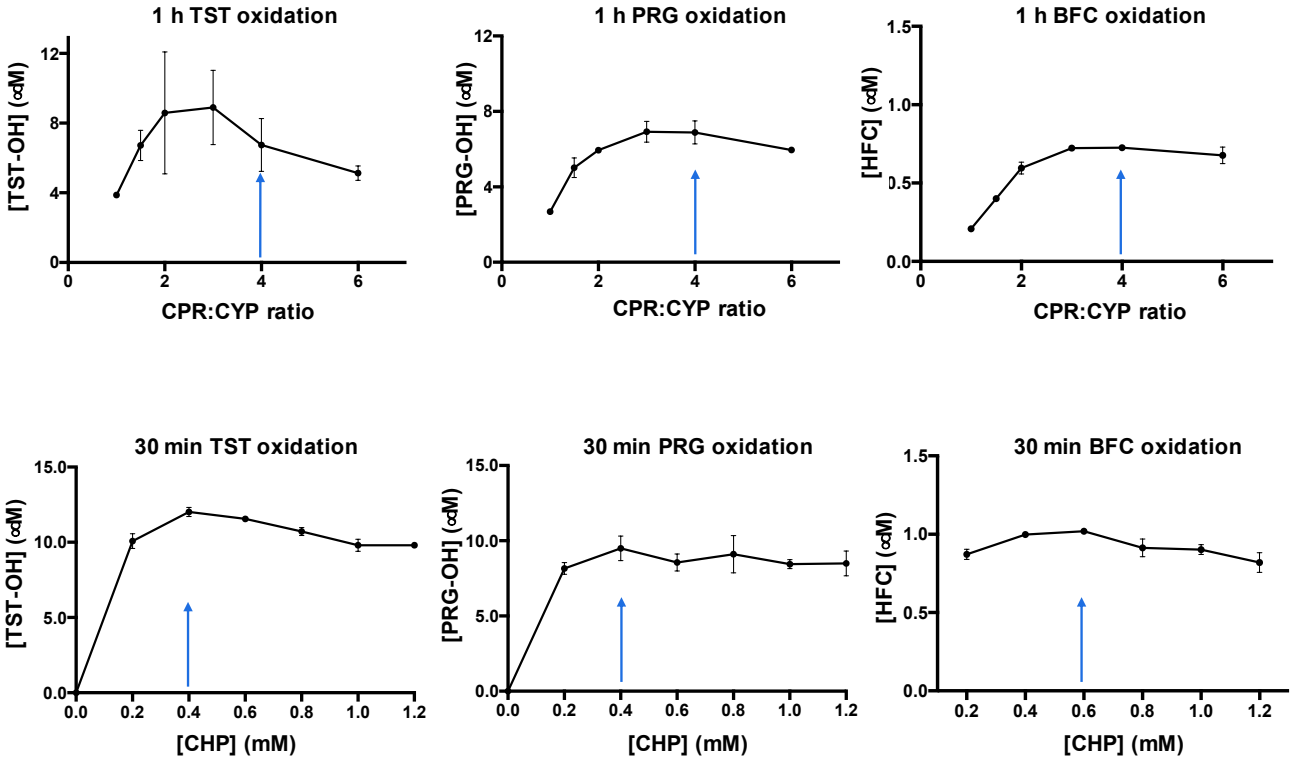


Figure S5. Concentration-dependent effect of CPR and CHP on the activity of CYP3A4 towards testosterone (TST), progesterone (PRG) and BFC. Error bars represent the standard deviations of duplicates or triplicate measurements.

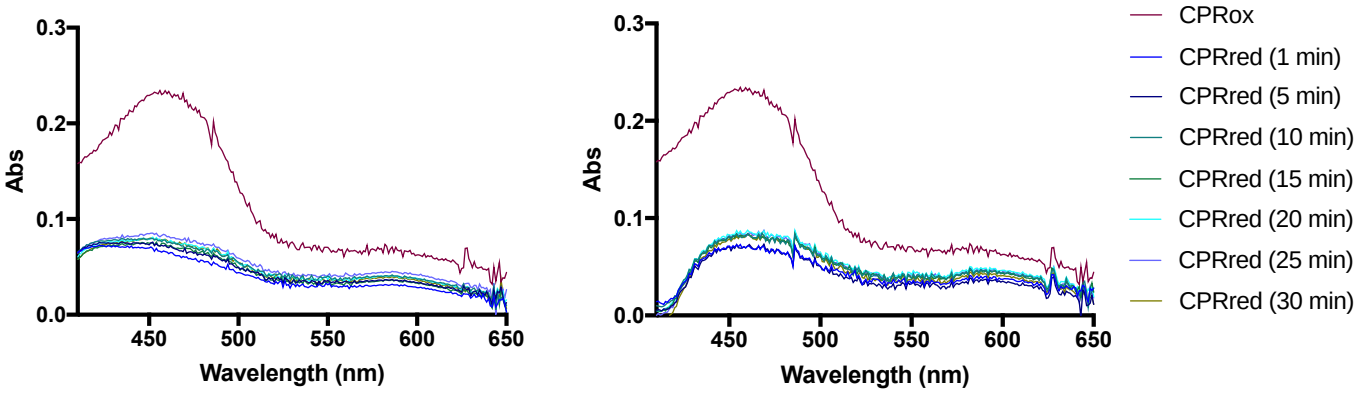


Figure S6. Absorption spectra of *ox*CPR and *red*CPR. The purple trace represents the spectrum of CPR in its oxidized form. Other CPR spectra were recorded at different time points (1-30 min) after addition of 1 mM NADPH. The reduction of CPR was measured both in the absence (left) and presence (right) of CYP3A4 (ratio 4:1 CPR to CYP3A4). Conditions: CYP3A4 (2.9 µM), CPR (11.7 µM), NADPH (1 mM), potassium phosphate buffer (0.1 M, pH 7.4, 10% glycerol) at room temperature.

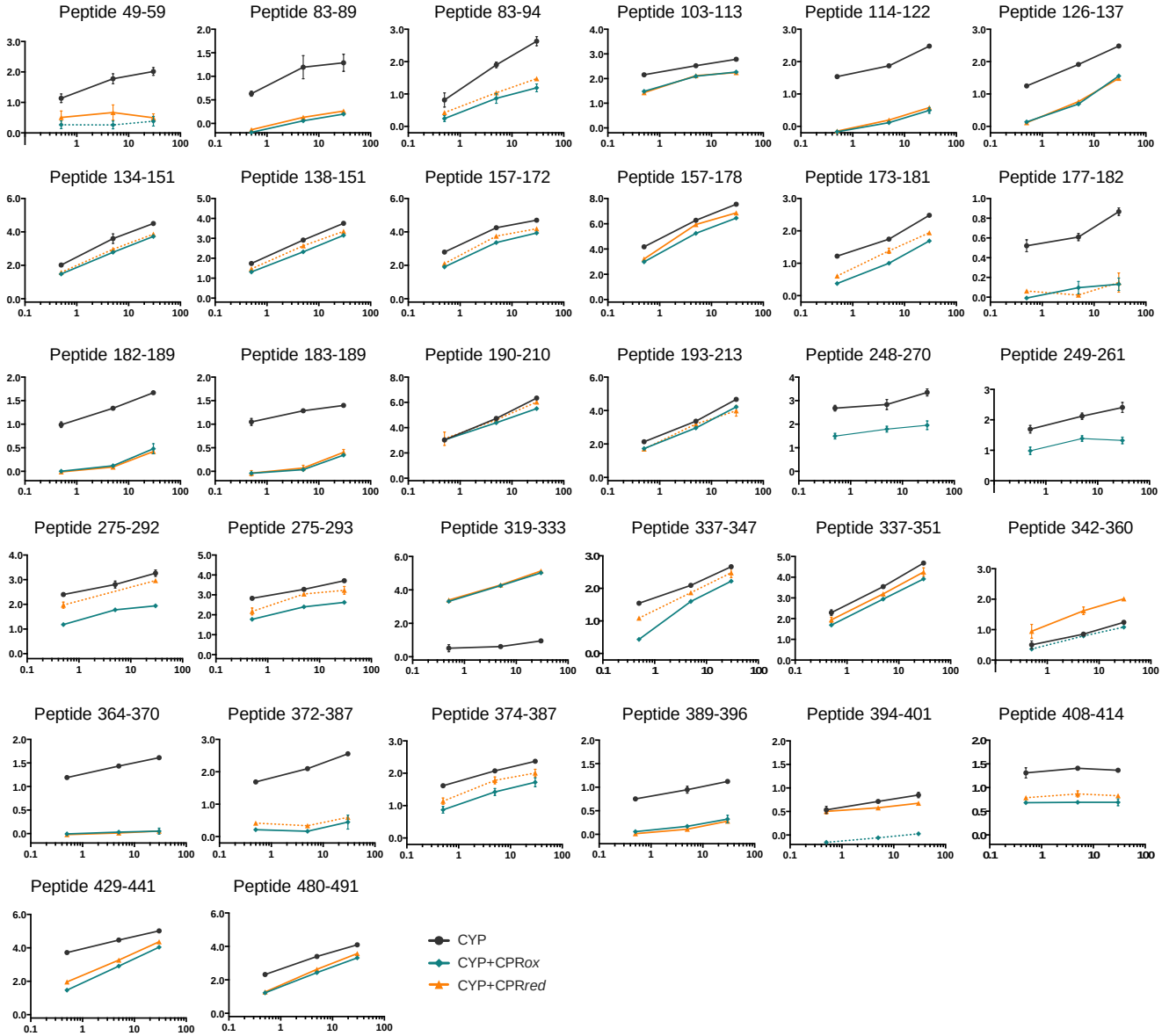


Figure S7. Deuterium uptake plots for all regions showing significant changes in the presence of either *ox*CPR or *red*CPR. The y-axis represents the deuterium uptake (Da) and the x-axis is time in a logarithmic scale. Black curves are for [CYP] alone, teal curves for [CYP:*ox*CPR], and orange curves for [CYP:*red*CPR]. Dashed curves indicate that the difference in deuterium uptake was not significant ([CYP] – [CYP:CPR*ox*] CI 99.9%: ± 0.60 Da; [CYP] – [CYP:CPR*red*] CI 99.9%: 0.69 ± Da). Peptides 240-270 and 249-261 were not detected in the CYP+CPR*red* state. Error bars represent the standard deviations of triplicate measurements.

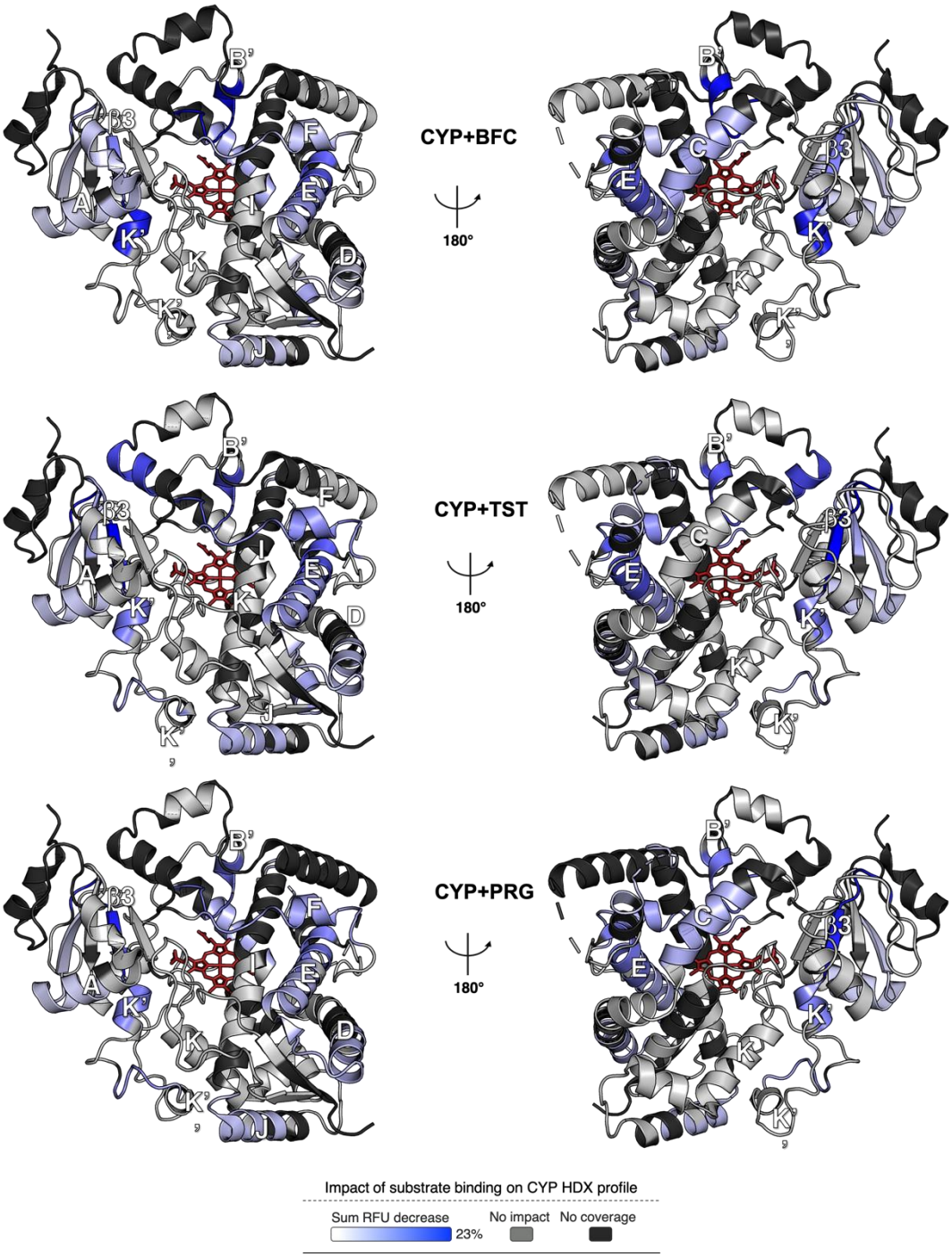


Figure S8. Impact of substrate binding on the structural dynamics of CYP3A4. Differential HDX profiles comparing [CYP] minus [CYP:BFC], [CYP:TST] or [CYP:PRG] are shown. The significant sum RFU difference (CI 99%: ± 0.34 Da (BFC), ± 0.36 Da (TST) and ± 0.45 Da (PRG)) of two time points (5, 60 min) is mapped onto the CYP3A4 structure (PDB: 1W0F), shown in both distal (left) and proximal (right) views. The blue color indicates a segment undergoing a decrease in deuterium uptake upon interaction with the substrate. The light gray color reveals regions unaffected by substrate binding, whereas black is used to show regions non-covered in the MS analysis.

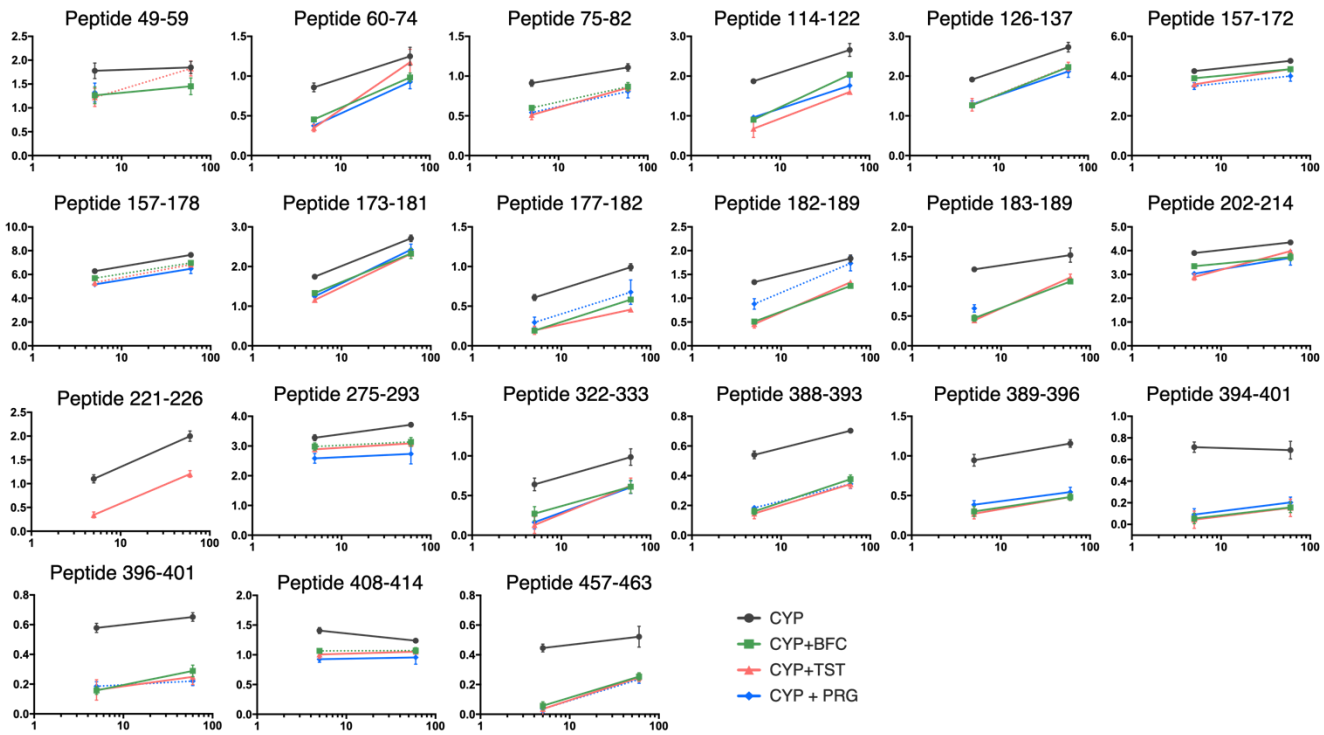


Figure S9. Deuterium uptake plots for all regions showing a significant change in the presence of substrate. The y-axis represents the deuterium uptake (Da) and the x-axis is time in a logarithmic scale. Black curves are for CYP alone; green, pink and blue curves are for CYP in the presence of BFC, TST and PRG, respectively. Dashed curves indicate that the difference in deuterium uptake between CYP was not significant ([CYP] – [CYP:BFC] CI 99%: ± 0.34 Da; [CYP] – [CYP:TST] CI 99%: 0.36 ± Da; [CYP] – [CYP:PRG] CI 99%: ± 0.45 Da). Peptide 221-226 was not detected in [CYP:BFC] and [CYP:PRG] states. Error bars represent the standard deviations of triplicate measurements.

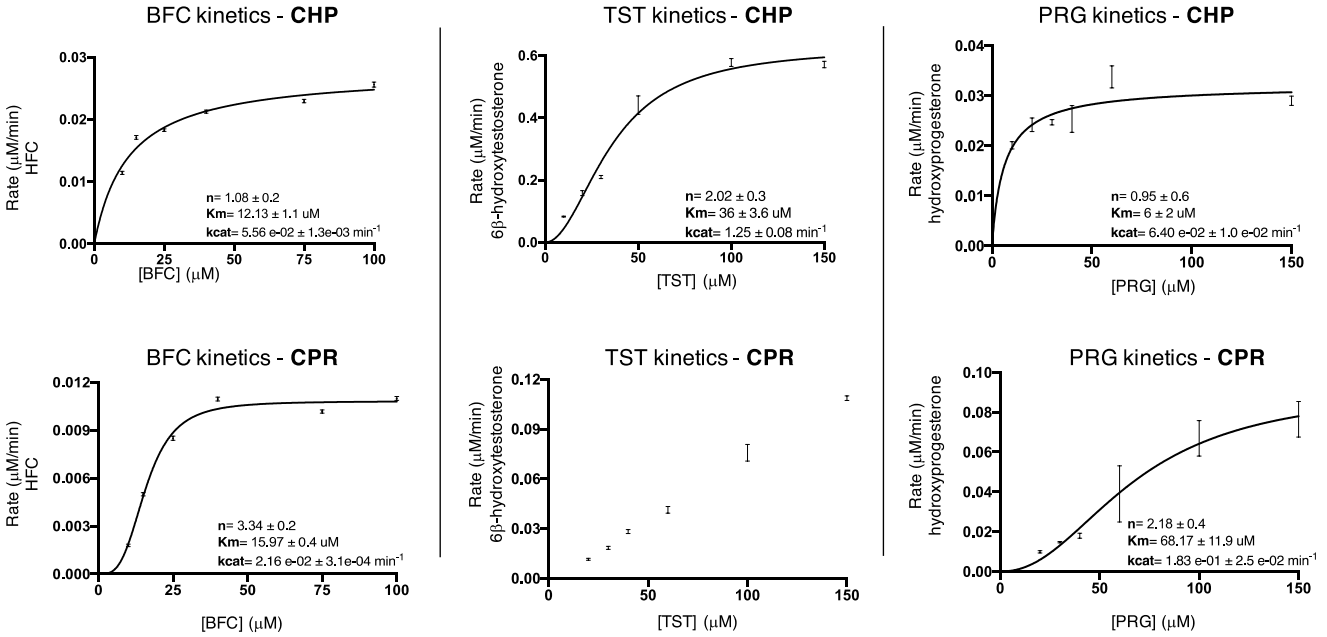


Figure S10. Impact of CPR on the functional cooperativity and activity of CYP3A4. Kinetics of BFC, TST and PRG oxidation by CYP3A4 (0.5 μM) was measured in the presence of CPR (2 μM) or CHP (0.4-0.6 mM). Error bars represent the standard deviations of duplicate or triplicate measurements. Globally, enzyme kinetics with CHP is faster and more hyperbolic. Due to poor solubility of steroids in aqueous solutions and the low catalytic turnover of the CPR-supported system, V_{max} for TST hydroxylation could not be reached, precluding an accurate kinetic characterization of this system. The faster rates achieved with CHP, however, allowed us to calculate steady state kinetic constants.

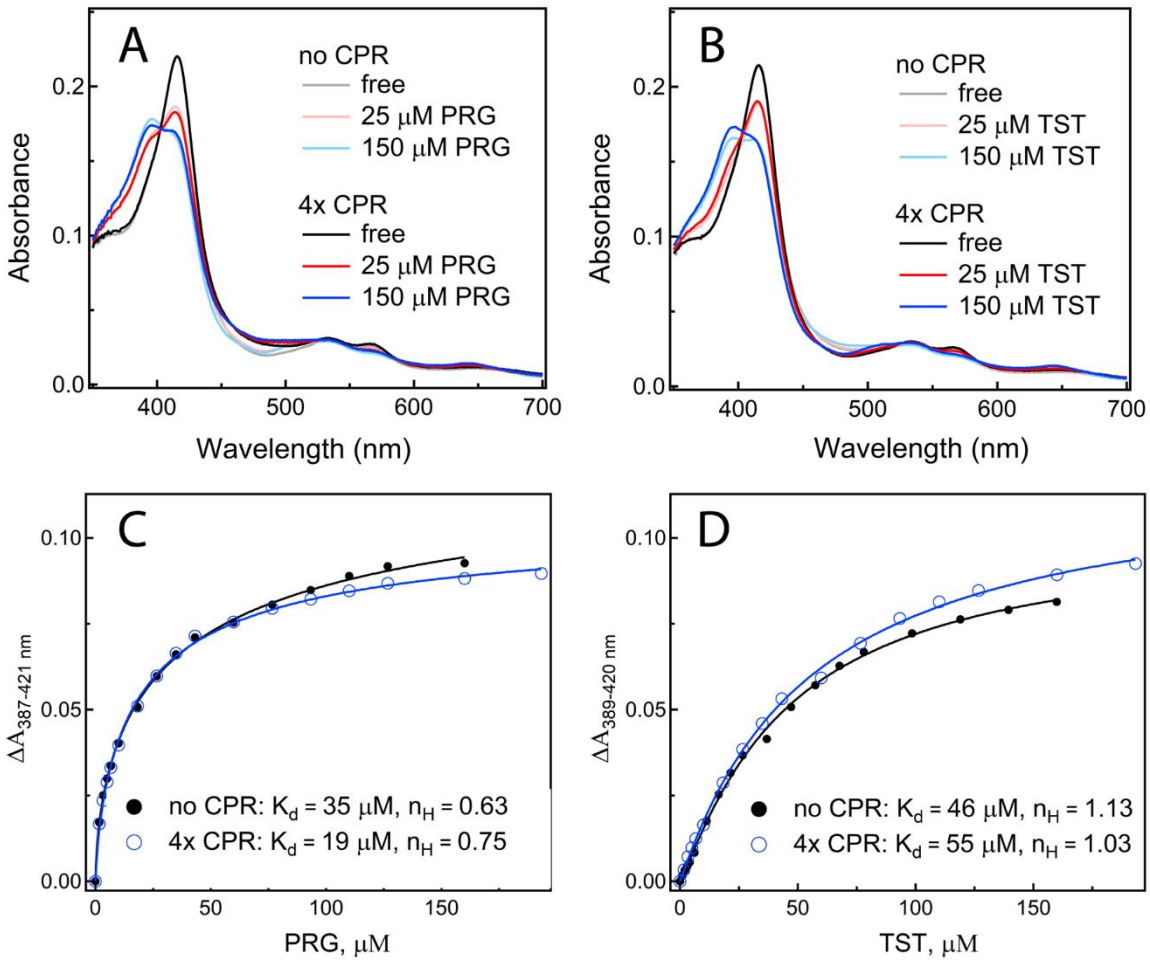


Figure S11. The impact of CPR on the spectral properties of CYP3A4 and its binding to substrate. **A** and **B**, absorption spectra of CYP3A4 (1.5 μM) in the absence and presence of variable concentrations of PRG/TST and a four-fold molar excess *ox*CPR (6 μM). Substrate addition shifts the heme absorption from 421 nm to 387 nm, indicative of the low-to-high spin transition in the heme iron. **C** and **D**, titration plots for PRG and TST, respectively, fitted with the Hill equation. The K_d and n_H values derived from the fits are shown in the table.

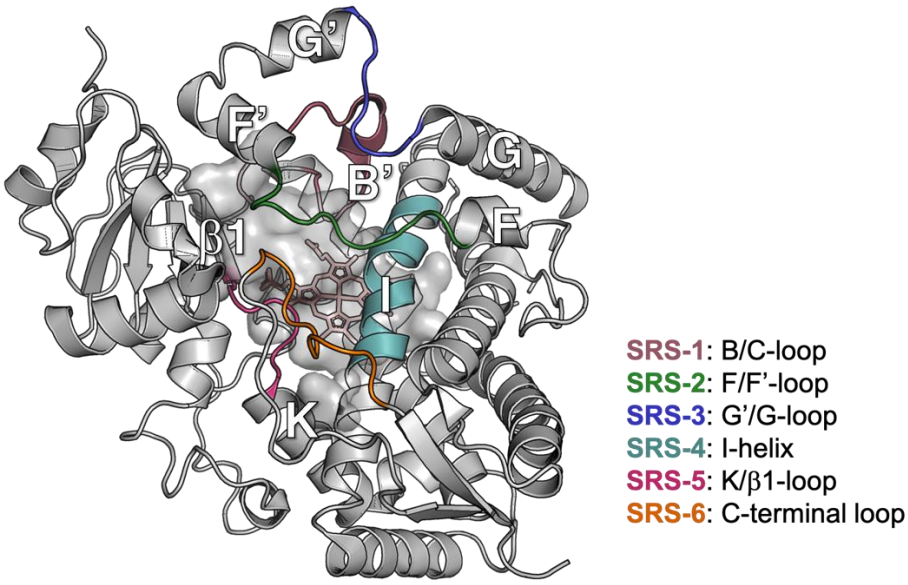
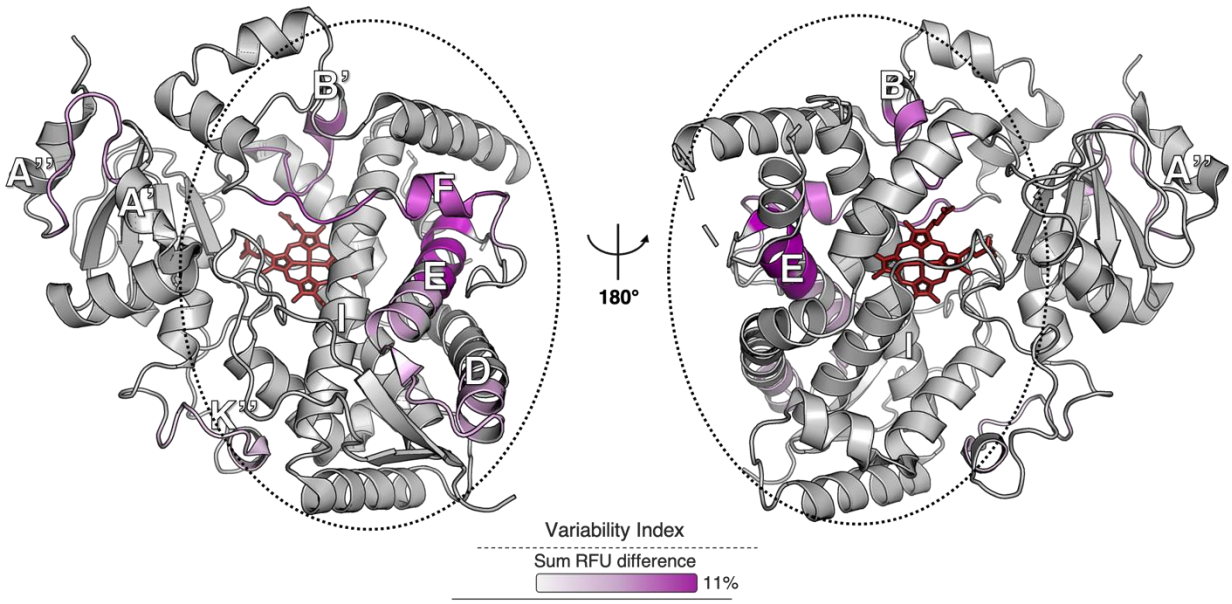


Figure S12. The substrate recognition sites (SRSs) of CYPs mapped onto the distal side of CYP3A4 structure (1W0F). The six SRS regions are highlighted: SRS-1; raspberry (B/C-loop), SRS-2; green (F/F′-loop), SRS-3; marine (G′/G-loop), SRS-4; teal (I-helix), SRS-5; pink (K/β1-loop), SRS-6; orange (C-terminal loop). SRS-4 and SRS-5 are deeper in the active site, running just over the heme group. Substrate bind to the distal side of the heme group. The active site surface is displayed in light gray.



Sum of DU and RFU differences between [CYP + substrate] states

Peptide	BFC – PRG[1]	BFC – TST[2]	TST – PRG[3]	Variability Index[4]
A''/A'-loop; 49-59	-0.38 Da; 4% RFU			4 ± 1 %
B'-helix; 114-122	0.44 Da; 6% RFU			6 ± 0.9 %
D/E-loop; 157-172	0.31 Da; 2% RFU			5 ± 0.3 %
D/E-loop; 157-178		0.55 Da; 3% RFU		3 ± 1 %
E-helix; 182-189		-0.36 Da; 5% RFU	-0.43 Da; 6% RFU	11 ± 3 %
F-helix; 193-210		-0.41 Da; 3% RFU		3 ± 0.7 %
F-helix; 202-214	0.48 Da; 4% RFU	0.32 Da; 3% RFU		7 ± 2 %
H/I-loop; 275-293		0.42 Da; 2% RFU		2 ± 1 %
K''-helix; 408-428		0.35 Da; 2% RFU		2 ± 0.9 %

[1] Negative sign denotes more rigidity in the presence of BFC. CI 95%: 0.27 Da.
[2] Negative sign denotes more rigidity in the presence of BFC. CI 95%: 0.22 Da.
[3] Negative sign denotes more rigidity in the presence of TST. CI 95%: 0.27 Da.
[4] Variability index is calculated based on the sum of the absolute sum RFU difference between all three states comparisons. The standard deviation of each RFU measurements was used for error propagation.

Figure S13. Variability in the structural dynamics of CYP3A4 upon binding to BFC, TST or PRG. Only those CYP3A4 peptides that show a significant deuterium uptake difference (CI 95%) upon binding to each of the three substrates are included in this analysis. The regions are mapped on the structure of CYP3A4 (PDB: 1W0F) in both distal (left) and proximal (right) views. In the data table, the relative uptake (in units of Da) is calculated according to the indicated difference. The relative fractional uptake (in units of %) is reported as the absolute value of the difference. The variability index is calculated as the sum of the absolute values of the RFU differences between each pair of substrate-bound states.