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

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SUPPORTING INFORMATION

Data Set	СҮРЗА4	CYP3A4 + rCPR <i>ox</i>	CYP3A4 + rCPR <i>red</i>
HDV reaction details	0.1 M KPi, pD = 7.0, RT,	0.1 M KPi, pD = 7.0, RT,	0.1 M KPi, pD = 7.0, RT, 1 μM
	1 μM CYP	1 μM CYP, 4μM CPR	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
UDV reaction datails	0.1 M KPi, pD = 7.0, RT,	0.1 M KPi, pD = 7.0, RT,	0.1 M KPi, pD = 7.0, RT,
HDX reaction details	1 μM CYP, 100 μM BFC	1 μM CYP, 100 μM TST	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.

		15	20	25	30	35	40	45	50	55	60	65	70	75	80
		0.57		120.01			10.55	0550		15151		100		0.00	100
LAITDP	DMIKT	VLVKE	CYSVF	TNRRP	FGPVG	FMKSA	ISIAE	DEEWK	RLRSL	LSPTF	TSGKL	KEMVF	ILAQY	GDVL	RNL
85	90	95	100	105	110	115	120	125	130	135	140	145	150	155	16
2	- /	-		2						-				8	-
REAETG	KPVTL	KDVFG	A Y SMD	VITST	SFGVN	IDSLN	INPODE	FVENT	KKLLF	FDFLD	PFFLS	ITVFF	FLIPI	LEVL	IICV
165	170	175	180	185	190	195	200	205	210	215	220	225	230	235	24
										10					
		-	-												
											1000			10.0	
PREVTN	FLRKS	VKRMK	E SRLE	ртокн			SQNSK	CE TE SH	KALSE	LELVA		IFAGY	ETTS	SVL SF I	MYE
PREVTN 245	IFLRKS 250	VKRMK 255	ESRLE 260	DTQKH 265	270	QLM10 275	280	CE TE SH 285	KALSE 290	LELVA 295	Q S I I F 300	IFAGY 305	ETTSS 310	SVL SF 1 315	MYE 32
PREVTN 245	IFLRKS 250	VKRMK 255	E SRLE 260	DTQKH 265	IRVDFL 270	275) SQ N SH 280	(E T E SH 285	KA L S D 290	LELVA 295	Q S I I F 300	IFAGY 305	2ETTSS 310	SVL SF 315	MYE 32
PREVTN 245	250	V K RMK 255	E SRLE 260	DTQKH 265	270	275	280 SQN SH	(E T E SH 285	290	LELVA 295	QSIIF 300	1 FAGY 305	2 E T T S S 310	SVL SF 1 315	MYE 32
PREVTN 245 ATHPDV		V K RMK 255 E E I DA	E SRLE 260	DTQKH 265 APPTY			280 280	285	PIAME	LELVA 295	QSIIF 300	I FAGY 305	YETTSS 310	SVLSF1 315	MYE 32
PREVTN 245 ATHPDV 325	250 250 200 200 200 200 200 200 200 200	V K RMK 255 E E I DA 335	E SRLE 260 VLPNK 340	DTQKH 265 APPTY 345	270 270 DTVLQ 350	QLM10 275 MEYLC 355) SQN SH 280 MVV NE 360	TLRLF	290 290 P I AMF 370	LELVA 295 LERVC 375	QSIIF 300 KKDVE 380	I FAGY 305 I NGMF 385	2 E T T S S 310 I P K G V 390	315 7VVMIF 395	MYE 32 SYA 40
PREVTN 245 ATHPDV 325	250 200 QQKLQ 330	255 EEIDA 335	E SRLE 260 VLPNK 340	DTQKH 265 APPTY 345	DTVLQ	QLMIE 275 MEYLE 355	280 280 0MVV NE 360	ETESH 285 ETLRLF 365	290 P I AMF 370	LELVA 295 LERVC 375	QSIIF 300 KKDVE 380	IFAGY 305 INGMF 385	210 310 I PKGV 390	315 315 /VVM I F 395	MYE 320 SYA 400
PREVTN 245 ATHPDV 325	250 250 200 200 200 200 200 200 200 200	VKRMK 255 E E I DA 335	E SRLE 260 VLPNK 340	DTQKH 265 APPTY 345	DTVLQ 350	QLMIE 275 MEYLE 355	280 280 MVV NE 360	ETESH 285 TLRLF 365	290 P I AMF 370	LELVA 295 LERVC 375	QSIIF 300 KKDVE 380	I FAGY 305 I NGMF 385	210 310 1 PKGV 390	SVL SF I 315 /VVM I F 395	MYE 320 SYA 400
PREVTN 245 ATHPDV 325	IFLRKS 250 QQKLQ 330	EEIDA 335	E SRLE 260 VLPNK 340	DTQKH 265 APPTY 345			280 280 0MVV NE 360	ETLRLF 365	PIAMF 370	LE LVA 295 LE RVC 375	QSIIF 300 KKDVE 380	I FAGY 305 I NGMF 385		SVL SF I 315 /VVM I F 395	SLG
PREVTN 245 ATHPDV 325 HRDPKY	VQQKLQ 330	VKRMK 255 EEIDA 335 E KFLPE 415	E SRLE 260 VLPNK 340 RF SKK 420	DTQKH 265 APPTY 345 NKDNI 425		ACLMIC 275 MEYLC 355 TPFGS 435	280 280 0MVV NE 360 6G P R NC 440	ETLRLF 365 IGMRF	PIAMF 370 ALMNN 450	LE LVA 295 LE RVC 375	QSIIF 300 KKDVE 380 RVLQN 460	I FAGY 305 I NGMF 385	210 210 210 210 210 200 200 200 200 200	315 7VVM1F 395	SLG
PREVTN 245 ATHPDV 325 HRDPKY 405	UF L R K S 250 VQQ K L Q 330 WT E P E 410	VKRMK 255 EEIDA 335 KFLPE 415	E SRLE 260 VLPNK 340 RF SKK 420	DTQKH 265 APPTY 345 NKDNI 425	RVDFL 270 7DTVLQ 350 DPY1Y 430	QLM1E 275 MEYLE 355 TPFGS 435	0 SQN SH 280 0MVV NE 360 6G P R NC 440	ETLRLF 365 CIGMRF 445	P I AMR 370 A LMNN 450	LELVA 295 LERVC 375 KLALI 455	QSIIF 300 KKDVE 380 RVLQN 460	I FAGY 305 I NGMF 385 F SF KF 465	2 E T T S S 310 F I PKGV 390 C KE TG 470	SVLSF1 315 /VVM1F 395 395 31PLKL 475	SYA 400 .SLG 480
PREVTN 245 ATHPDV 325 HRDPKY 405	VQQKLQ 330 WTEPE 410	VKRMK 255 EEIDA 335 KFLPE 415 VESRD	E SRLE 260 VLPNK 340 RF SKK 420 GTV SG	DTQKH 265 APPTY 345 NKDN I 425 AHHHH	DPY I Y	QLMIE 275 MEYLE 355 TPFGS 435	0 SQN SH 280 0MV/V NE 360 SG P R NC 440	ETLRLF 365 CIGMRF 445	PIAMF 370 ALMNN 450	LELVA 295 LERVC 375 KLALI 455	RVLQN 460 RCYP+C	I FAGY 305 I NGMF 385 F SF KF 465 PR <i>ox</i>]; :	PCKETG 470 29 pepti	SVLSFI 315 VVVMIF 395 21PLKL 475	SYA 400 .SLG
PREVTN 245 ATHPDV 325 HRDPKY 405 SLLQPEK 485	VQQKLQ 330 WTEPE 410 CPVVLK 490	VKRMK 255 EEIDA 335 KFLPE 415 VESRD 495	E SRLE 260 VLPNK 340 RF SKK 420 GTV SG 500	DTQKH 265 APPTY 345 NKDNI 425 AHHHH 505	DPY I Y 430	QLMIE 275 MEYLE 355 TPFGS 435	0 SQN SH 280 0MVV NE 360 SG P R NC 440	ETLRLF 285 ETLRLF 365 ETLRLF 445	P I AMF 370 A LMNN 450	LELVA 295 LERVC 375 KLALI 455	RVLQN 460 RCYP+C	I FAGY 305 I NGMF 385 F SF KF 465	PC KE TC 470 29 pepti	SVLSF1 315 7VVM1F 395 31PLKL 475 ides 	SYA 40 SLG 48

Deute	Deuterium uptake difference: [CYP:CYP+CPRox]							
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					

			15	20	25	30	35	40	45	50	55	60	65	70	75	8
VLA	ITDP	DMIKT	VLVKE	CYSVF	TNRRP	FGPVG	FMKSA	ISIA	DEEW	RLRSI	LSPTE	TSGKL	KEMVP	IIAQY	GDVLV	RNL
	85	90	95	100	105	110	115	120	125	130	135	140	145	150	155	16
	AFTG		KDVEG	AYSME	VITST	SEGVI		NPOD	PEVENT			PFFIS	ITVEP	FIIPI	LEVIN	
	165	170	175	180	185	190	195	200	205	210	215	220	225	230	235	24
FPRI	FVTN	FIRKS	VKRMK	FSRIF	ртокн	RVDFI	OIMIC	SONSI	(FTF SI	IKAI SI		OSLIF	LEAGY	FTTSS	VI SE I	MYF
	245	250	255	260	265	270	275	280	285	290	295	300	305	310	315	32
LATI	HPDV	QQKLQ	EEIDA	VLPNK	APPTY	DTVLG	MEYLD			PIAME	RLERVO	KKDVE	INGMF	I PKGV		SYA
	325	330	335	340	345	350	355	360	365	370	375	380	385	390	395	40
LHR	DPKY	WTEPE	KFLPE	RFSKK	NKDNI	DPYIN	TPFGS	GPRNO	GMR	ALMIN	IKLAL	RVLQN	FSFKP	CKETQ	IPLKL	SLO
8	405	410	415	420	425	430	435	440	445	450	455	460	465	470	475	48
										[0	CYP:CYI	P+CPRre	ed]; 15 p	peptide	s	
ci i i			VESDO	CTVSC					-3.3	7 Da 🖂					+3.7 Da	a
GLL	485	490	495	500	505					32	.2% co	verage, s	5 min e	kposure	2	

Deuterium uptake difference [CYP:CYP+CPRred]							
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+CPRox:CYP+CPRred]							
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).

MALLLAVFLVLLYLYGTHSHGLFKKLGIPGPTPLPFLGNILSYHKGFCMFDMECHKKYGKVWGFYDGQQP 15 20 25 30 35 40 45 50 55 60 65 70 75 80 VLA I TDPDM I KTVLVKECY SVFTNRRPFGPVGFMKSA I SIAE DE EWKRLR SLL SPTFT SGKLKEMVP I I AQYGDVLVRNL 85 90 95 100 105 110 115 120 125 130 135 140 145 150 155 160 RREAETGKPVTLKDVFGAYSMDVITSTSFGVNIDSLNNPQDPFVENTKKLLRFDFLDPFFLSITVFPFLIPILEVLNICV 165 170 175 180 185 190 195 200 205 210 215 220 225 230 235 240 FPREVTNFLRKSVKRMKESRLEDTQKHRVDFLQLMIDSQNSKETESHKALSDLELVAQSIIFIFAGYETTSSVLSFIMYE 245 250 255 260 265 270 275 280 285 290 295 300 305 310 315 320 LATHPDVQQKLQEE I DAVLPNKAPPTYDTVLQMEYLDMVVNETLRLFPIAMRLERVCKKDVE I NGMF I PKGVVVM I PSYA 325 330 335 340 345 350 355 360 365 370 375 380 385 390 395 400 **x** LHRDPKYWTEPEKFLPERFSKKNKDNIDPYIYTPFGSGPRNCIGMRFALMNMKLALIRVLQNFSFKPCKETQIPLKLSLG 405 410 415 420 425 430 435 440 445 450 455 460 465 470 475 48(GLLQPE KPVVL KVE SRDGTV SGAHHHH 485 490 495 500 505 25.4% coverage, 60 min exposure

Deuterium untake difference: [CVP:CVP+BEC]								
Deuten								
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					

MALLLAVFLVLLYLYGTHSHGLFKKLGIPGPTPLPFLGNILSYHKGFCMFDMECHKKYGKVWGFYDGQQP 15 20 25 30 35 40 45 50 55 60 65 70 75 80 VLAITDPDMIKTVLVKECYSVFTNRRPFGPVGFMKSAISIAEDEEWKRLRSLLSPTFTSGKLKEMVPIIAQYGDVLVRNL _____85 90 95 100 105 110 115 120 125 130 135 140 145 150 155 16C
 RREAE TGKPVTLKDVFGAY SMDV IT ST SFGVN I D SLNNPQDPFVENTKKLLRF DFLDPFFLS ITVF PFLIPILEVLNICV

 165
 170
 175
 180
 195
 200
 205
 210
 215
 220
 225
 230
 235
 240
 165 170 1
 FPREVTNFLRK SVKRMKE SRLEDTQKHRVDFLQLMID SQN SKETE SHKAL SDLELVAQSI I FIFAGYETT SSVL SF IMYE

 245
 250
 265
 270
 275
 280
 285
 290
 295
 300
 315
 315
 320
 LATHPDVQQKLQEEIDAVLPNKAPPTYDTVLQMEYLDMVVNETLRLFPIAMRLERVCKKDVEINGMFIPKGVVVMIPSYA 325 330 335 340 345 350 355 360 365 370 375 380 385 390 395 400 LHRDPKYWTE PEKFLPERF SKKNKDN I DPY I YTPFG SGPRNC I GMRFALMNMKLAL I RVLQNF SFKPCKE TQ I PLKL SLG 405 410 415 420 425 430 435 440 445 450 455 460 465 470 475 480 [CYP:CYP+TST]; 15 peptides GLLQPEKPVVLKVESRDGTVSGAHHHH 485 490 495 500 505 -1.1 Da 🗔 🗔 +1.1 Da 28.8% coverage, 60 min exposure

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					

		MA	LLLAV	FLVLI	YLYGT	HSHGL	FKKLG	IPGPT	PLPFI	LGNILS	YHKG	CMF DW	IECHKK	YGKW	VGFYD	GQQP
			15	20	25	30	35	40	45	50	55	60	65	70	75	80
														-	_	
VLAI	TDP	DMIKT	VLVKE	CYSVE	TNRRP	FGPVC	FMKSA	ISIAE	DEEW	KRLRSL	LSPT	TSGKL	KEMVF	ILAQ	GDVL	VRNL
8	85	90	95	100	105	110	115	120	125	130	135	140	145	150	155	16
RREA	ETG	KPVTL	KDVFG	AYSM	VITST	SFGVI	IDSLN	NPQDE	FVENT	TKKLLF	FDFL	PFFLS		FLIP	LEVL	NICV
1	65	170	175	180	185	190	195	200	205	210	215	220	225	230	235	24
FPRE	VTN	FLRKS	VKRMK	ESRLE	DTQKH	RVDFL	QLMID	SQNS	ETE SH	HKALSE	LELVA	QSIIF	IFAGY	ETTS	SVLSF	IMYE
2	45	250	255	260	265	270	275	280	285	290	295	300	305	310	315	32
LATH	PDV		EEIDA	VLPN	APPTY	DTVLO	MEYLD	MVVNE		F P I AMF	LERVO	KKDVE	INGME	I PKG		PSYA
3	25	330	335	340	345	350	355	360	365	370	375	380	385	390	395	40
LHRD	PKY	WTEPE	KFLPE	RFSK	(NKDN I	DPYIN	TPFGS	GPRNO	GMR	FALMIN	IKLAL	RVLQN	IFSFKF	CKETO	PLK	LSLG
4	05	410	415	420	425	430	435	440	445	450	455	460	465	470	475	48
	-	2														
											[CYP:0	CYP+PR	G]; 12 p	eptides	5	_
6110	PEK	PVVLK	VESRD	GTVS	БАНННН	1				-1.2 D	a				+1.2[Da
ULLU				2010						2	C CO/ -					

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%: \pm 0.34 Da; [CYP] – [CYP:TST] CI 99%: 0.36 \pm Da; [CYP] – [CYP+PRG] CI 99%: \pm 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:oxCPR] (CI 99.9%); B) [CYP] – [CYP:redCPR] (CI 99.9%); C) [CYP:oxCPR] – [CYP:redCPR] (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%: \pm 0.60 Da; [CYP] – [CYP:CPR*red*] CI 99.9%: 0.69 \pm 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%: \pm 0.34 Da (BFC), \pm 0.36 Da (TST) and \pm 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%: \pm 0.34 Da; [CYP] – [CYP:TST] CI 99%: 0.36 \pm Da; [CYP] – [CYP:PRG] CI 99%: \pm 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 μ M) was measured in the presence of CPR (2 μ M) 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 μ M) in the absence and presence of variable concentrations of PRG/TST and a four-fold molar excess *ox*CPR (6 μ M). 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/ β 1-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. Cl 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.