Supporting Information:

Evidence of allosteric coupling between substrate binding and Adx recognition in the vitamin-D carbon-24 hydroxylase CYP24A1

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with Adx		
Peptide	**Peptide Mass**	**pLink Score**
GLMIL**E**GQEW(6) -GIG**K**RM(4)	1833.934901	6.32 E-03
GLMILEGQ**E**W(9) -GIG**K**RM(4)	1833.934901	1.66 E-05 2.14 E-05 1.36 E-02
ILEGQ**E**W(6) -GIG**K**RM(4)	1532.788903	2.35 E-03
DIVATDNEPV**E**ML(11) -GIG**K**RM(4)	2120.036117	4.08 E-06
LQEVQSVLP**D**NQTPRAEDL(10) -GIG**K**RM(4)	2810.446383	8.80 E-03
Q**K**ETEEEALTF(2) -**E**KRFGLL(1)	2168.123276	2.37 E-04 6.02 E-03 8.58 E-03 2.07 E-02 4.51 E-02
TLNTQVLGSSEDNF**E**DSHKF(15) -SELN**K**W(5)	3025.41725	6.54 E-02
SF**E**SICLVLY(3) -SELN**K**W(5)	1987.983289	7.30 E-06 9.15 E-03
SQQPGADFLCDIYQQ**D**HL(16) -SK**K**ELY(3)	2883.361656	1.19 E-02
CDIYQQ**D**HL(7) -SK**K**ELY(3)	1939.921752	5.38 E-03
TLNTQVLGSS**E**DNF(11) -EDSH**K**FRPERW(5)	2992.418254	1.49 E-04 5.86 E-02
TLNTQVLGSSE**D**NF(12) -EDSH**K**FRPERW(5)	2992.418254	4.92 E-09 1.22 E-08
DSVHLGSPSLL(1) -RM**K**LGSF(3)	1960.031966	6.38 E-04

Adx withheld		
Peptide	**Peptide Mass**	**pLink Score**
SF**E**SICLVLY(3) -SELN**K**W(5)	1987.983289	4.31 E-09
CDIYQQ**D**HL(7) -S**K**KELY(2)	1939.921752	3.47 E-05
LCDIYQQ**D**HL(8) -S**K**KELY(2)	2053.00581	1.91 E-10 4.25 E-05 5.68 E-05
LCDIYQQ**D**HL(8) -SK**K**ELY(3)	2053.00581	2.80 E-02
SQQPGADFLCDIYQQ**D**HL(16) -S**K**KELY(2)	2883.361656	6.55 E-10 1.90 E04
TLNTQVLGSSE**D**NF(12) -EDSH**K**FRPERW(5)	2992.418254	2.58 E-08 2.60 E07
DSVHLGSPSLL(1) -RM**K**LGSF(3)	1960.031966	1.95 E-03

Table S1. **Differential EDC cross-linking of CYP24A1 in response to Adx**. Adx was found to induce formation of additional cross-linked peptides when compared to a control condition in which CYP24A1 is incubated with EDC and Adx is withheld (right panel). CYP24A1 peptides are listed in the table with Adx (left panel) and Adx withheld (right panel). Data were analyzed using pLink

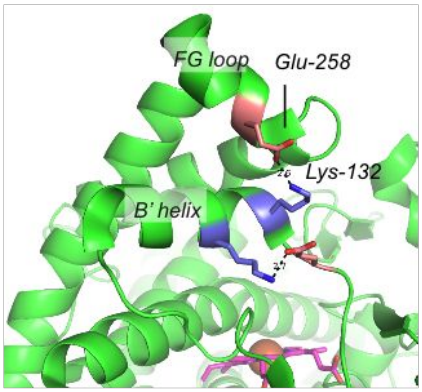
software (pLink scores are shown in the right hand column). Identified peptide products that correspond to the same region in CYP24A1 are grouped together.

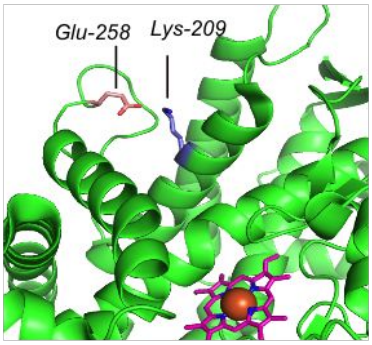
Peptide	$\frac{Intensity\ (CYP24A1 + Adx)}{Intensity\ (CYP24A1 + Adx + 1,25(OH)_2 D3)}$ Ratio	pValue
^{72}EIFWKGGLKKQHDTL^{86}	0.325	0.017
^{280}KSVKPCIDNRLQRY^{293}	0.259	0.004
^{163}QKKLMKPVEIMKL^{175}	0.111	0.009
^{220}YEKRFGLLQKETEEEALTF^{238}	0.000	0.017

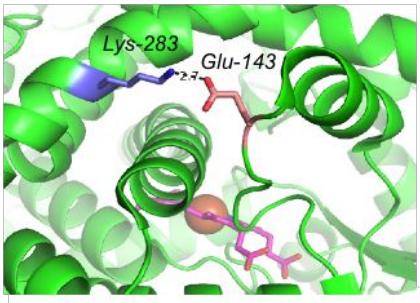
Table S2. **Substrate-induced EDC/LC-MS pattern of CYP24A1 cross-linking**. Data were generated by comparative analysis of the signal intensities of peptides produced by chymotrypsin digestion of CYP24A1 in the presence of Adx and in the presence or absence of 1,25(OH)$_2$D3. Peptides were quantified as a ratio from EDC cross-linked samples in triplicate, in the absence of substrate compared to the same experiment upon addition of substrate. The addition of 1,25(OH)$_2$D3 alone produced no change in EDC reactivity (no peptides were differentially affected). However, the addition of 1,25(OH)$_2$D3 into samples that also contained Adx (and compared against this same condition without substrate) resulted in identification of four peptides, corresponding to the F and G helices, the A-helix,

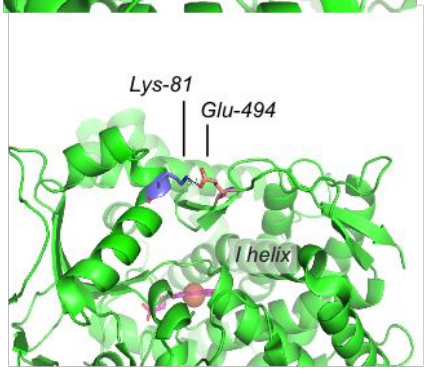
and the C-D helical bend on CYP24A1 (Figure 4 in the main body of the manuscript).

CYP24A1, PDB 3K9V
0 ns

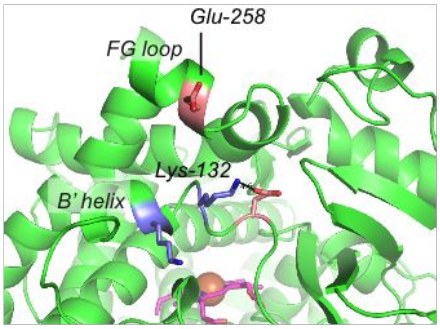


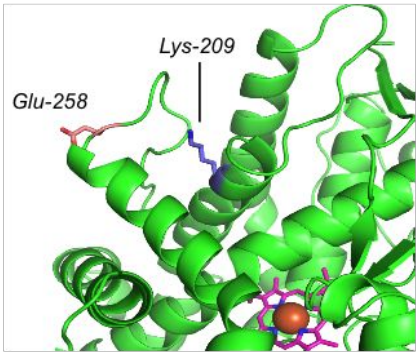


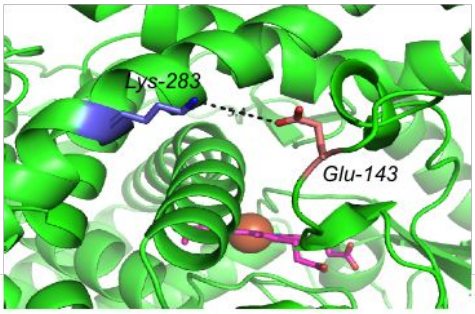




CYP24A1$^{154-459}$
25 ns







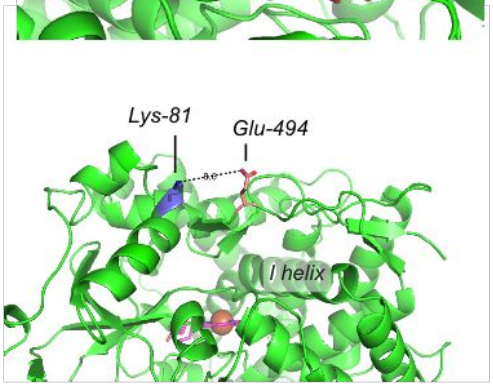


Figure S1. Mapping of native salt-bridge interactions from substrate-affected regions in open and closed models of CYP24A1. Peptides differentially affected by addition of 1,25$(OH)_2$D3 (Table S2) map to the F, G, A, and C/D helices of

CYP24A1. A comparison of the open form of CYP24A1 (PDB ID 3K9V at 0 ns dynamics simulation) and the closed form ($CYP24A1^{154-459}$ at 25 ns simulation) reveals the presence of multiple sat bridge interactions within the identified peptides that are likely sensitive to open-to-close conformational transition of CYP24A1.

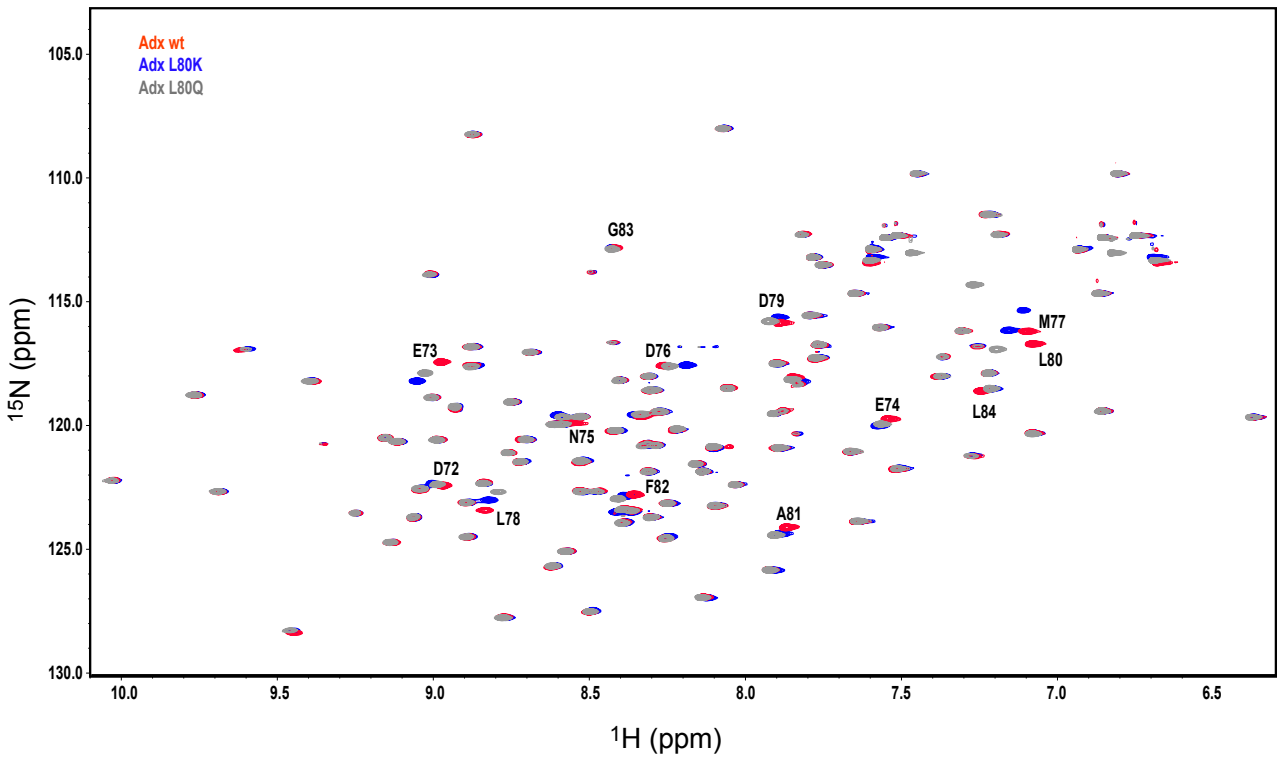


Figure S2. Overlay of ^{1}H-^{15}N HSQC spectra of WT and mutant Adx. Local amide chemical shift changes due to L80Q or L80K mutations. Spectra were acquired on samples containing 100 uM labeled protein concentration in 50 mM potassium phosphate, 50 mM NaCl, pH 6.9, and 5% D2O. Acquisition parameters consisted of 4 scans across 128 increments on a Bruker 800 MHz Avance spectrometer.