Supplementary Information
Distant residues mediate picomolar-binding affinity of a protein cofactor

Yves J.M. Bollen[1,2], Adrie H. Westphal[2], Simon Lindhoud[2], Willem J.H. van Berkel[2] and Carlo P.M. van Mierlo[2]*

[1]Department of Structural Biology, Vrije Universiteit, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands

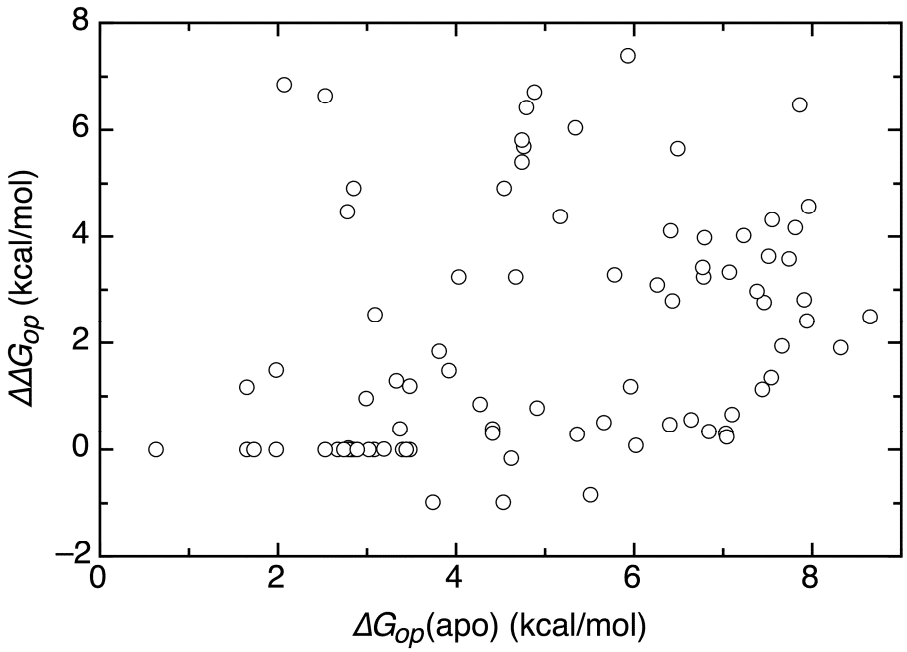
[2]Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

*Corresponding author

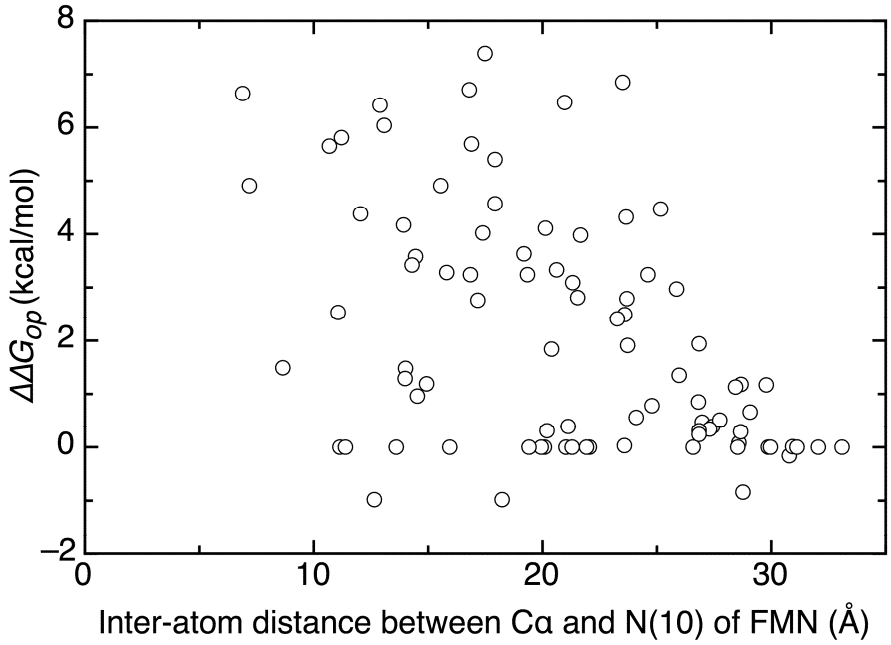
Phone: +31 317 484621

Fax: +31 317 484801

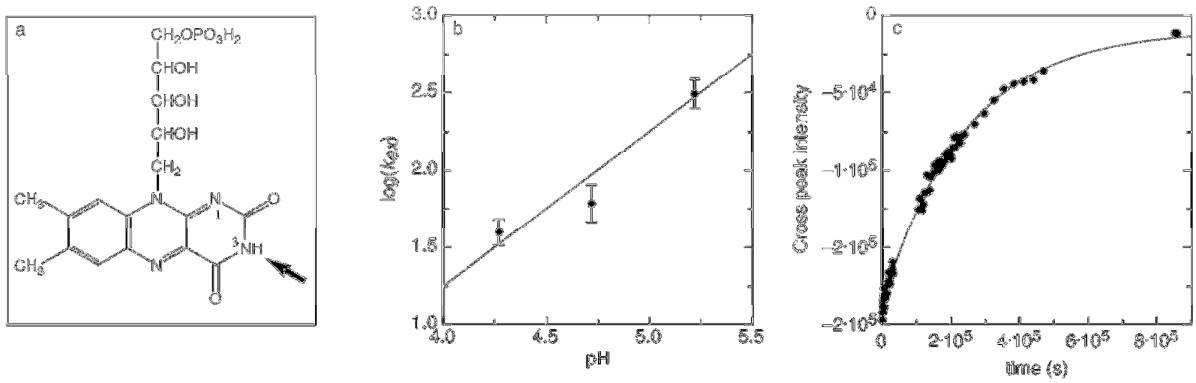
E-mail:carlo.vanmierlo@wur.nl



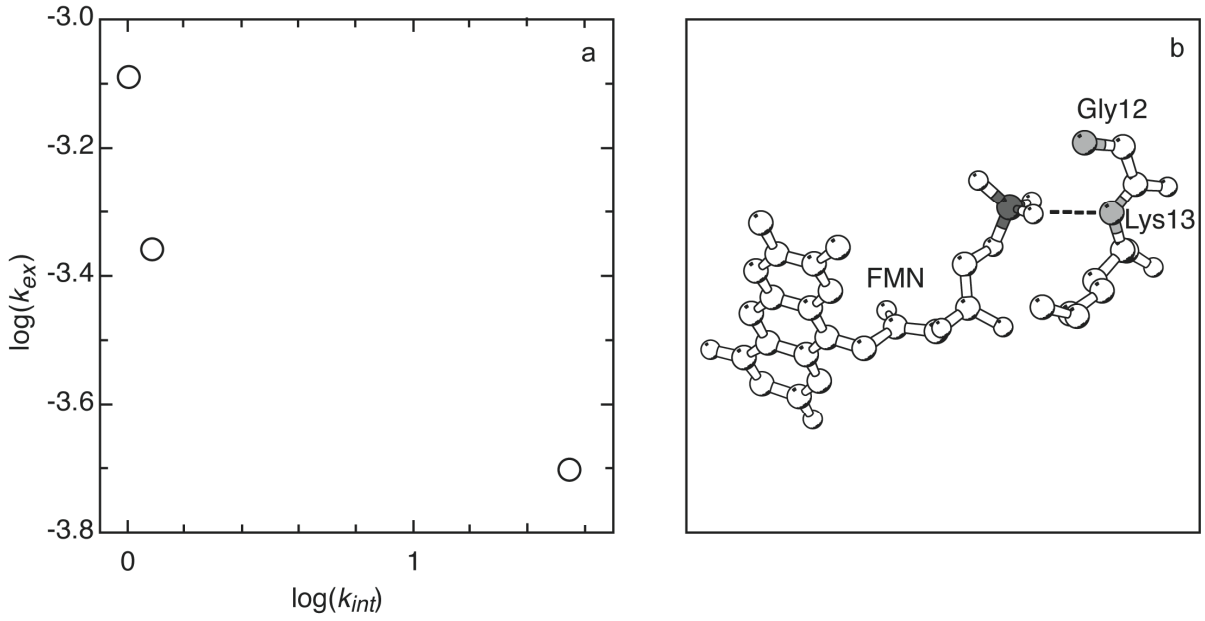
Supplementary Figure S1. The magnitudes of $\Delta\Delta G_{op}$-values do not correlate with ΔG_{op}(apoflavodoxin). The difference in ΔG_{op} at the residue level between apoflavodoxin and flavodoxin (i.e., $\Delta\Delta G_{op}$) as determined by H/D exchange at pD 6.3, 25 °C is plotted against the ΔG_{op}-value at that particular residue in apoflavodoxin.



Supplementary Figure S2. No significant correlation exists between the distance a residue has to FMN and $\Delta\Delta G_{op}$. The difference in ΔG_{op} at the residue level between apoflavodoxin and flavodoxin (i.e., $\Delta\Delta G_{op}$) as determined by H/D exchange at pD 6.3, 25 °C is plotted against the inter-atom distance between various Cα-atoms and N(10) of FMN in flavodoxin (using pdb ID 1YOB).



Supplementary Figure S3. Hydrogen exchange of N(3)H of flavodoxin's cofactor. (a) Structure of FMN, with N(3)H indicated. (b) Fitting of a linear equation with a fixed slope of 1 to the pH-dependent exchange rates of N(3)H of FMN at 1.0 °C shows that the data are described by: $\log(k_{ex}) = ((-2.75 \pm 0.05) + \text{pH})$.. Data points represent the rate constant as determined by fitting an exponential equation to selective saturation recovery NMR data, whereas error bars show the standard fitting error. In the linear fitting procedure, data points are weighted according to the standard error of the exponential fit. (c) H/D exchange curve obtained for N(3)H of flavodoxin at pD 6.3, 25 °C. Due to folding in the ^{15}N dimension of the ^{1}H-^{15}N HSQC experiment this intensity is negative. The exchange rate is $(3.803 \pm 0.007)\cdot 10^{-6}$ s^{-1}.



Supplementary Figure S4. Exchange of the backbone amide of Lys13 decreases upon increasing pD. (a) k_{ex} of Lys13 of flavodoxin as a function of intrinsic exchange rate, k_{int}. (b) The backbone amide of Lys13 is hydrogen bonded to the phosphate of FMN, as indicated by the dashed line. The graph is generated using Molscript[55].

Supplementary Table S1. Amide proton exchange rates, k_{ex}, and stabilities against local opening, ΔG_{op}, of flavodoxin (pD 6.26) and apoflavodoxin (pD 6.28). The proteins are in 100 mM KPPi, 25 °C.[a]

Residue		k_{ex}(flavodoxin)	ΔG_{op}(flavodoxin)	k_{ex}(apoflavodoxin)	ΔG_{op}(apoflavodoxin)	$\Delta\Delta G_{op}$
1	Ala	-	-	-	-	-
2	Lys	-	-	> 0.01	< 4.82	-
3	Ile	1.91 E-06	6.87	0.44 E-05	6.40	0.47
4	Gly	-	-	-	-	-
5	Leu	4.07 E-09	10.77	0.36 E-05	6.79	3.98
6	Phe	1.97 E-09	11.14	0.97 E-06	7.51	3.63
7	Phe	3.46 E-08 (pD 8.82)	13.29	-	-	-
8	Gly	7.87 E-09	11.38	0.23 E-03	5.34	6.04
9	Ser	-	-	-	-	-
10	Asn	> 0.01	< 3.72	-	-	-
11	Thr	> 0.01	< 2.98	-	-	-
12	Gly	-	-	-	-	-
13	Lys	4.37 E-04	4.67	-	-	-
14	Thr	4.33 E-06	7.28	-	-	-
15	Arg	5.44 E -07	8.82	-	-	-
16	Lys	7.16 E-07	8.52	-	-	-
17	Val	2.56 E-08	9.45	0.11 E-03	4.54	4.91
18	Ala	< 1 E-10	> 13.32	0.29 E-04	5.93	> 7.39
19	Lys	1.45 E-08	10.52	0.16 E-04	6.41	4.11
20	Ser	-	-	> 0.01	< 3.33	-
21	Ile	4.29 E-08	9.35	0.84 E-05	6.26	3.09
22	Lys	7.68 E-08	9.22	0.92 E-05	6.43	2.79
23	Lys	4.88 E-06	7.25	> 0.01	< 2.78	> 4.47
24	Arg	4.12 E-04	4.80	0.83 E-03	4.41	0.39
25	Phe	4.64 E-06	7.14	0.36 E-04	5.96	1.18
26	Asp	> 0.01	< 2.32	-	-	-
27	Asp	-	-	-	-	-
28	Glu	-	-	> 0.01	< 1.73	-
29	Thr	1.52 E-03 (pD 6.19)	3.36	-	-	-
30	Met	3.93 E-05	6.17	0.98 E-04	5.66	0.51
31	Ser	4.50 E-04	5.12	~ 2 E-03	~ 4.27	~ 0.85
32	Asp	-	-	> 0.01	< 2.67	-
33	Ala	9.87 E-04	3.76	~ 2 E-03	~ 3.37	~ 0.39
34	Leu	3.81 E-08	9.22	-	-	-
35	Asn	> 0.01	< 3.02	-	-	-
36	Val	-	-	-	-	-
37	Asn	5.89 E-05	6.15	-	-	-

Residue		k_{ex}(flavodoxin)	ΔG_{op}(flavodoxin)	k_{ex}(apoflavodoxin)	ΔG_{op}(apoflavodoxin)	$\Delta\Delta G_{op}$
38	Arg	1.85 E-03	4.18	-	-	-
39	Val	2.58 E-04	4.15	-	-	-
40	Ser	> 0.01	< 2.95	-	-	-
41	Ala	> 0.01	< 3.05	> 0.01	< 3.08	~ 0
42	Glu	> 0.01	< 1.95	> 0.01	< 1.98	~ 0
43	Asp	-	-	7.02 E-03	2.27	-
44	Phe	-	-	-	-	-
45	Ala	3.27 E-05	6.10	0.39 E-04	6.02	0.08
46	Gln	4.49 E-04 (pD 6.19)	4.46	0.42 E -03	4.62	-0.16
47	Tyr	2.75 E-04	4.67	0.69 E-04	5.51	-0.84
48	Gln	> 0.01	< 2.79	> 0.01	< 2.82	~ 0
49	Phe	4.14 E-06	7.18	0.78 E-05	6.84	0.34
50	Leu	1.69 E-09	11.14	0.12 E-06	8.65	2.49
51	Ile	2.23 E-09	10.40	0.66 E-06	7.07	3.33
52	Leu	7.11 E-10	11.25	0.68 E-06	7.23	4.02
53	Gly	1.53 E-09	11.98	0.19 E-05	7.81	4.17
54	Thr	2.03 E-09	11.87	-	-	-
55	Pro	-	-	-	-	-
56	Thr	2.04 E-07	8.59	-	-	-
57	Leu	6.57 E-04	3.73	-	-	-
58	Gly	1.01 E-03	4.08	-	-	-
59	Glu	> 0.01	< 2.18	-	-	-
60	Gly	8.64 E-04	4.25	-	-	-
61	Glu	3.98 E-04	4.08	-	-	-
62	Leu	~ 2 E-03	~ 2.60	-	-	-
63	Pro	-	-	-	-	-
64	Gly	1.54 E-03 (pD 6.19)	3.69	-	-	-
65	Leu	1.27 E-03	3.30	-	-	-
66	Ser	> 0.01	< 2.86	-	-	-
67	Ser	-	-	-	-	-
68	Asp	> 0.01	< 2.64	> 0.01	< 2.67	~ 0
69	Ala	-	-	> 0.01	< 2.42	-
70	Glu	> 0.01	< 1.95	-	-	-
71	Asn	> 0.01	< 3.10	-	-	-
72	Glu	> 0.01	< 2.38	-	-	-
73	Ser	1.97 E-04	5.26	-	-	-
74	Trp	2.04 E-04	4.79	-	-	-
75	Glu	2.43 E-04	3.99	-	-	-
76	Glu	1.73 E-05	5.50	-	-	-
77	Phe	7.53 E-07	7.71	-	-	-
78	Leu	-	-	-	-	-

Residue		k_{ex}(flavodoxin)	ΔG_{op}(flavodoxin)	k_{ex}(apoflavodoxin)	ΔG_{op}(apoflavodoxin)	$\Delta\Delta G_{op}$
79	Pro	-	-	-	-	-
80	Lys	6.63 E-06	6.58	-	-	-
81	Ile	9.14 E-09	10.02	0.23 E-05	6.78	3.24
82	Glu	1.03 E-05	5.69	0.41 E-04	4.91	0.78
83	Gly	> 0.01	< 2.80	> 0.01	< 2.83	~ 0
84	Leu	2.38 E-05	5.65	0.41 E-04	5.36	0.29
85	Asp	-	-	-	-	-
86	Phe	-	-	> 0.01	< 2.10	-
87	Ser	-	-	0.42 E-03	5.12	-
88	Gly	> 0.01	< 3.42	> 0.01	< 3.44	~ 0
89	Lys	2.32 E-06	7.76	0.74 E-05	7.10	0.66
90	Thr	-	-	0.37 E-05	7.40	-
91	Val	6.74 E-09	10.35	0.42 E-06	7.94	2.41
92	Ala	6.50 E-09 (pD 8.82)	14.33	0.11 E-05	7.86	6.47
93	Leu	6.93 E-09	10.22	0.78 E-06	7.46	2.76
94	Phe	1.46 E-09	11.32	0.66 E-06	7.74	3.58
95	Gly	2.18 E-09	12.14	0.33 E-04	6.49	5.65
96	Leu	1.67 E-07 (pD 8.82)	12.05	-	-	-
97	Gly	-	-	> 0.01	< 2.75	-
98	Asp	2.49 E-07	8.72	-	-	-
99	Gln	2.25 E-08	10.15	-	-	-
100	Val	2.63 E-06 (pD 7.73)	8.82	-	-	-
101	Gly	2.32 E-06	7.76	> 0.01	< 2.85	> 4.91
102	Tyr	1.25 E-07	9.17	> 0.01	< 2.53	> 6.64
103	Pro	-	-	-	-	-
104	Glu	> 0.01	< 1.62	> 0.01	< 1.65	~ 0
105	Asn	8.01 E-05	5.95	-	-	-
106	Tyr	3.91 E-08	10.06	-	-	-
107	Leu	> 0.01	< 1.92	-	-	-
108	Asp	5.68 E-07 (pD 8.82)	11.19	-	-	-
109	Ala	-	-	-	-	-
110	Leu	2.15 E-08	9.55	0.38 E-04	5.17	4.38
111	Gly	3.18 E-08	10.19	0.11 E-04	6.77	3.42
112	Glu	2.76 E-04	4.30	-	-	-
113	Leu	3.50 E-08	9.06	0.96 E-05	5.78	3.28
114	Tyr	6.35 E-08 (pD 8.82)	12.53	0.42 E-06	7.96	4.57
115	Ser	-	-	0.60 E-05	7.62	-
116	Phe	-	-	0.13 E-05	8.03	-
117	Phe	-	-	0.57 E-06	8.19	-
118	Lys	2.74 E-08	10.23	0.73 E-06	8.32	1.91
119	Asp	3.85 E-04	4.32	-	-	-

Residue		k_{ex}(flavodoxin)	ΔG_{op}(flavodoxin)	k_{ex}(apoflavodoxin)	ΔG_{op}(apoflavodoxin)	$\Delta\Delta G_{op}$
120	Arg	2.83 E-06	7.33	0.49 E-05	7.03	0.31
121	Gly	5.24 E-04 (pD 6.19)	4.95	-	-	-
122	Ala	-	-	-	-	-
123	Lys	3.49 E-06	7.28	0.55 E-05	7.04	0.24
124	Ile	1.83 E-03	2.82	~ 2 E-03	~ 2.79	~ 0.03
125	Val	5.16 E-07	7.20	0.14 E-05	6.64	0.56
126	Gly	> 0.01	< 2.82	> 0.01	< 2.85	~ 0
127	Ser	> 0.01	< 3.38	> 0.01	< 3.40	~ 0
128	Trp	1.02 E-06	7.91	0.26 E-03	4.67	3.24
129	Ser	> 0.01	< 3.00	> 0.01	< 3.02	~ 0
130	Thr	-	-	~ 2 E-03	~ 3.93	-
131	Asp	1.71 E-03	3.55	~ 2 E-03	~ 3.48	~ 0.07
132	Gly	> 0.01	< 2.76	~ 2 E-03	~ 3.74	> -0.98
133	Tyr	2.56 E-04	4.67	~ 2 E-03	~ 3.48	~ 1.19
134	Glu	3.78 E-04	3.95	~ 2 E-03	~ 2.99	~ 0.96
135	Phe	1.81 E-03	3.12	-	-	-
136	Glu	-	-	~ 2 E-03	~ 3.01	-
137	Ser	1.54 E-04	5.40	~ 2 E-03	~ 3.92	~ 1.48
138	Ser	> 0.01	< 3.55	~ 2 E-03	~ 4.53	> -0.98
139	Glu	2.14 E-04	4.62	~ 2 E-03	~ 3.33	~ 1.29
140	Ala	-	-	0.49 E-03	4.24	-
141	Val	7.86 E-07	7.27	0.20 E-03	4.03	3.24
142	Val	8.76 E-06	5.65	0.21 E-03	3.81	1.84
143	Asp	7.45 E-08 (pD 6.19)	8.92	> 0.01	< 2.07	> 6.85
144	Gly	> 0.01	< 2.76	> 0.01	< 2.79	~ 0
145	Lys	4.01 E-04	4.72	0.71 E-03	4.41	0.31
146	Phe	1.43 E-08	10.42	0.22 E-03	4.76	5.69
147	Val	6.92 E-09	10.14	0.69 E-04	4.74	5.40
148	Gly	3.47 E-09	11.59	0.32 E-03	4.88	6.71
149	Leu	-	-	-	-	
150	Ala	3.04 E-09	11.21	0.17 E-03	4.79	6.42
151	Leu	3.96 E-09	10.55	0.78 E-04	4.74	5.81
152	Asp	> 0.01	< 1.95	-	-	-
153	Leu	9.93 E-04	2.97	-	-	-
154	Asp	7.54 E-04	3.47	> 0.01	< 1.98	> 1.49
155	Asn	1.31 E-04	5.62	> 0.01	< 3.09	> 2.53
156	Gln	-	-	-	-	-
157	Ser	> 0.01	< 3.42	> 0.01	< 3.44	~ 0
158	Gly	-	-	-	-	-
159	Lys	> 0.01	< 2.82	-	-	-
160	Thr	> 0.01	< 2.71	> 0.01	< 2.74	~ 0

Residue		k_{ex}(flavodoxin)	ΔG_{op}(flavodoxin)	k_{ex}(apoflavodoxin)	ΔG_{op}(apoflavodoxin)	$\Delta\Delta G_{op}$
161	Asp	> 0.01	< 2.51	> 0.01	< 2.53	~ 0
162	Glu	> 0.01	< 1.70	> 0.01	< 1.73	~ 0
163	Arg	1.09 E-05	6.57	-	-	-
164	Val	-	-	0.14 E-04	5.89	-
165	Ala	-	-	0.95 E-05	6.58	-
166	Ala	-	-	0.28 E-04	6.14	-
167	Trp	1.31 E-07 (pD 7.73)	10.72	0.54 E-06	7.91	2.81
168	Leu	1.19 E-07 (pD 8.82)	11.87	0.52 E-06	7.55	4.32
169	Ala	4.63 E-08	9.60	0.13 E-05	7.66	1.94
170	Gln	2.87 E-07	8.89	0.30 E-05	7.54	1.35
171	Ile	6.27 E-09	10.35	0.10 E-05	7.38	2.97
172	Ala	2.57 E-07	8.57	0.18 E-05	7.44	1.13
173	Pro	-	-	-	-	-
174	Glu	1.08 E-03 (pD 6.19)	2.82	> 0.01	< 1.65	> 1.17
175	Phe	2.60 E-06	6.98	-	-	-
176	Gly	-	-	-	-	-
177	Leu	1.57 E-03	3.18	0.16 E-02	3.19	0.01
178	Ser	> 0.01	< 2.86	> 0.01	< 2.89	~ 0
179	Leu	> 0.01	< 0.60	> 0.01	< 0.63	~ 0

[a] Rate constants for H/D exchange are in s^{-1}. Local stabilities against opening of flavodoxin (ΔG_{op}(flavodoxin)) and of apoflavodoxin (ΔG_{op}(apoflavodoxin)), as well as the differences in local stabilities between both proteins ($\Delta\Delta G_{op}$; $\Delta\Delta G_{op}$ = (ΔG_{op}(flavodoxin) - ΔG_{op}(apoflavodoxin)) are in kcal/mol. The standard deviation of ΔG_{op} is ± 0.35 kcal/mol (see Materials and Methods) and thus the standard deviation in $\Delta\Delta G_{op}$ determination is calculated to be 0.50 kcal/mol. In case of rapidly exchanging amide protons that are not observed in the first HSQC spectrum of the protein in ^2H_2O, k_{ex} is set to be > 0.01 s^{-1}. Exchange rates of residues 41, 42, 48, 68, 83, 88, 104, 126, 127, 129, 144, 157, 160, 161, 162, 178 and 179 of both proteins are > 0.01 s^{-1}. For each of these residues upper limits of the corresponding ΔG_{op}-values are calculated and as these limits are virtually identical for both proteins the corresponding $\Delta\Delta G_{op}$ is set to ~ 0. In case of residues showing backbone amide cross peak intensities in the first two HSQC spectra acquired but displaying no such intensities in the third HSQC spectrum, k_{ex} is estimated to be ~ 2 E-03 s^{-1}. A (-) indicates that the exchange rate could not be determined, which is due to either unassigned cross peaks, overlapping cross peaks, or the residue being a proline. In case of some residues changing pD resolves cross peak overlap and as a result k_{ex} can be determined and thus is reported (for example, k_{ex} = 3.46 E-08 (pD 8.82) is k_{ex} obtained at pD 8.82). Equation S7, together with the appropriate k_{int}-values, has been used to calculate all ΔG_{op}-values.

Supplementary Table S2. Dependence of H/D exchange rates, k_{ex}, of backbone amides of flavodoxin on pD.[a] The corresponding rates for local opening, k_{op}, and closing, k_{cl}, of the protein structure are calculated as described in the main text.[b] Buffer is 100 mM KPPi, temperature is 25 °C.

Residue	k_{ex}(6.19)	k_{ex}(6.26)	k_{ex}(7.73)	k_{ex}(8.82)	k_{op}	k_{cl}
3	8.29E-07	1.91E-06				
5	no exch.	4.07E-09	6.56E-07	2.90E-06		
6	no exch.	1.97E-09	no exch.	1.15E-08	1.16E-08	1.57
7	no exch.	no exch.		3.46E-08		
8	no exch.	7.87E-09	8.89E-07	2.79E-06		
13	8.13E-04	4.37E-04	1.98E-04			
14	4.60E-06	4.33E-06	3.19E-05	2.11E-04	1.67E-04 ± 1.12E-04	41.75 ± 37.28
15		5.44E-07	4.17E-05	1.56E-04		
16	4.70E-07	7.16E-07	2.42E-04			
17	no exch.	2.56E-08	1.02E-05	4.65E-05		
19	no exch.	1.45E-08	2.85E-06	1.80E-05		
21		4.29E-08	1.22E-05	8.65E-05		
22	6.85E-08	7.68E-08	3.26E-05	1.97E-04		
23	1.80E-06	4.88E-06				
24	1.34E-04	4.12E-04				
25	1.94E-06	4.64E-06				
29	1.52E-03					
30	1.34E-05	3.93E-05				
31	1.56E-04	4.50E-04				
33	8.48E-04	9.87E-04				
34	8.11E-08	3.81E-08	8.03E-06	3.75E-05		
37	2.11E-05	5.89E-05				
38	7.67E-04	1.85E-03				
39	8.29E-05	2.58E-04				
45	1.06E-05	3.27E-05				
46	4.49E-04					
47	4.89E-05	2.75E-04				
49	1.70E-06	4.14E-06				
50	no exch.	1.69E-09	no exch.	1.08E-08		
51	no exch.	2.23E-09		8.87E-09	7.59E-09 ± 2.01E-09*	0.84 ± 0.44*
52	no exch.	7.11 E-10	no exch.	6.29E-09	7.59E-09 ± 2.01E-09*	0.84 ± 0.44*
53	no exch.	1.53E-09		1.04E-08	7.59E-09 ± 2.01E-09*	0.84 ± 0.44*
54	no exch.	2.03E-09	no exch.	2.64E-08		
56	8.90E-08	2.04E-07	1.15E-06	4.73E-06	3.90E-06 ± 2.09E-06	12.79 ± 9.36
57	6.85E-04	6.57E-04				
58	4.76E-04	1.01E-03				
60	6.15E-04	8.64E-04				
61		3.98E-04				

Residue	k_{ex}(6.19)	k_{ex}(6.26)	k_{ex}(7.73)	k_{ex}(8.82)	k_{op}	k_{cl}
64	1.54E-03					
65	8.32E-04	1.27E-03				
73	2.52E-04	1.97E-04				
74	1.90E-04	2.04E-04				
75		2.43E-04				
76		1.73E-05				
77	5.70E-07	7.53E-07	1.59E-04			
80	5.20E-06	6.63E-06				
81	no exch.	9.14E-09	2.79E-06	1.36E-05		
82	4.97E-06	1.03E-05				
84	7.97E-06	2.38E-05				
89	1.04E-06	2.32E-06				
91	no exch.	6.74E-09	2.20E-06	9.28E-06		
92	no exch.	no exch.	no exch.	6.50E-09	7.59E-09 ± 2.01E-09*	0.84 ± 0.44*
93	no exch.	6.93E-09	9.72E-07	4.42E-06		
94	no exch.	1.46E-09	no exch.	8.76E-09	7.59E-09 ± 2.01E-09*	0.84 ± 0.44*
95	no exch.	2.18E-09	no exch.	2.12E-07		
96	no exch.	no exch.	no exch.	1.67E-07		
98	1.18E-07	2.49E-07	3.33E-07	3.31E-07	3.43E-07 ± 8.20E-08	0.63 ± 0.42
99		2.25E-08	3.35E-06	2.15E-05		
100	no exch.		2.63E-06	1.19E-05		
101	2.70E-06	2.32E-06	1.03E-04			
102	no exch.	1.25E-07	2.88E-07	4.65E-07	3.84E-07 ± 8.46E-08	1.52 ± 0.85
105	3.77E-05	8.01E-05				
106	no exch.	3.91E-08	9.35E-06	5.45E-05		
108		no exch.	3.88E-07	5.68E-07		
110	no exch.	2.15E-08	4.43E-06	2.20E-05		
111		3.18E-08	6.97E-06	4.52E-05		
112	1.46E-04	2.76E-04				
113	1.56E-07	3.50E-08				
114	no exch.	no exch.	no exch.	6.35E-08		
118	no exch.	2.74E-08	1.01E-05	6.24E-05		
119	2.95E-04	3.85E-04				
120	1.41E-06	2.83E-06				
121	5.24E-04					
123	1.45E-06	3.49E-06				
124	5.47E-04	1.83E-03				
125	3.75E-07	5.16E-07	1.80E-04			
128	4.92E-07	1.02E-06	4.52E-04			
131	9.23E-04	1.71E-03				
133		2.56E-04				

Residue	k_{ex}(6.19)	k_{ex}(6.26)	k_{ex}(7.73)	k_{ex}(8.82)	k_{op}	k_{cl}
134	1.28E-04	3.78E-04				
135	8.37E-04	1.81E-03				
137	6.04E-05	1.54E-04				
139	2.67E-04	2.14E-04				
141	4.23E-07	7.86E-07	4.15E-04			
142	3.11E-06	8.76E-06				
143	7.45E-08					
145		4.01E-04				
146	no exch.	1.43E-08	5.63E-06	3.13E-05		
147	no exch.	6.92E-09	2.34E-06	1.16E-05		
148	no exch.	3.47E-09	1.46E-07	2.89E-07	4.04E-07 ± 2.46E-07	106.9 ± 89.45
150	no exch.	3.04E-09	4.00E-07	2.91E-07		
151		3.96E-09				
153	2.59E-04	9.93E-04				
154		7.54E-04				
155	4.90E-05	1.31E-04				
163	3.85E-06	1.09E-05				
167	no exch.	no exch.	1.31E-07	5.12E-07		
168	no exch.	no exch.	no exch.	1.19E-07		
169		4.63E-08	2.06E-05	9.20E-05		
170	1.83E-07	2.87E-07	1.66E-04			
171		6.27E-09	4.26E-06	2.53E-05		
172		2.57E-07				
174	1.08E-03					
175	1.26E-06	2.60E-06				
177	5.12E-04	1.57E-03				

[a] Rate constants for H/D exchange of flavodoxin are in s^{-1}. Backbone amide protons that show no exchange during the time course of an exchange experiment at a particular pD-value are indicated with "no exch.". Residues that are not incorporated in the table either have backbone amide protons that show no exchange at all pD-values used, or exchange too rapidly to follow by NMR spectroscopy at all pD-values used, or have unassigned ^{1}H-^{15}N cross peaks, or have overlapping ^{1}H-^{15}N cross peaks, or are prolines. Experiments are done at the specific pD-value indicated. For example, k_{ex}(6.19) is the H/D exchange rate determined in a native state exchange experiment at pD 6.19 (uncorrected pH-meter reading). Protein concentration ranges between 2.5 and 3 mM.

[b] Opening and closing rates (in s^{-1}) are determined by fitting equation S4 to the curving dependence of k_{ex} on pD. Rates marked with * are determined by globally fitting this equation to the pD-dependent exchange data of Ile 51, Leu 52, Gly 53, Ala 92 and Phe 94 (see main text for details). Errors indicated are standard errors. In case of residues where less than 3 k_{ex}-values are available no such error is given, since it cannot be determined.

Supplementary Methods

Materials. Potassium pyrophosphate (KPPi) was from Sigma (Bornem, Belgium). ^{15}N Ammonium chloride was purchased from Campro Scientific, Veenendaal, The Netherlands.

Native state H/D exchange. All equilibrium and kinetic folding experiments involving apoflavodoxin and flavodoxin were done at 25 °C using 100 mM KPPi as buffer[30-32]. Consequently, H/D exchange of the backbone amides of flavodoxin was measured using these sample conditions. To follow H/D exchange, lyophilized ^{15}N-labelled flavodoxin was dissolved into D_2O to protein concentrations ranging from 2.5 to 3 mM in 100 mM KPPi at various pD-values (pD is the uncorrected pH meter reading of a deuterated solution). Each sample was immediately transferred into the NMR machine and subsequently a series of ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) spectra was recorded. Similarly prepared samples without protein were used to shim the magnetic field, tune the probe, and determine the length of the 90° proton pulse prior to the start of the H/D exchange experiment. The dead time of an exchange experiment (i.e., the difference in time between mixing D_2O with lyophilized protein and recording of the first HSQC spectrum) was approximately 5 minutes. pD was measured after finishing the exchange experiments. pD-values of the samples used were 6.19, 6.26, 7.73 and 8.82. The H/D exchange experiments done at pD 6.26 and 8.82 lasted 11 months and at pD 6.19 and 7.73 the experiments lasted 2 months. Similar to the above procedure, H/D exchange data were also recorded of 1.3 mM apoflavodoxin in 100 mM KPPi at pD 6.28 during an 8-week period. For the purpose of NMR spectral referencing, all protein samples contained 100 μM 3-(trimethylsilyl)-1-propane sulfonic acid (DSS).

NMR spectroscopy and data processing. Gradient-enhanced ^{1}H-^{15}N HSQC spectra[50,51] were recorded on a Bruker AMX 500 MHz machine. In all exchange experiments, the first 9 HSQC spectra were recorded with 1024 complex data points in the ^{1}H dimension and 96 complex data points in the ^{15}N dimension, using two scans per increment. Each HSQC spectrum took 8 minutes and 40 seconds to acquire. Subsequently, spectra with identical settings but with 256 complex data points in the indirect dimension were recorded. Now, each HSQC experiment lasted 22 minutes and 46 seconds. Sample temperature was 25 °C, as verified using 4% methanol in d_4-methanol.

Spectra were processed using Felix 2D (MSI, San Diego, CA, USA). In the direct ^{1}H dimension, data were multiplied by a Gaussian function and zero filled to 2048 points prior to

Fourier transform. In the indirect ^{15}N dimension data were zero filled once and multiplied by a squared cosine bell prior to Fourier transform. Assignments were adapted from [49] by a pD titration and temperature alteration to the conditions used here. Maximal intensities of backbone amide cross peaks were determined using macros and scripts provided by A. G. Palmer III (http://www.palmer.hs.columbia.edu/software.html).

Hydrogen exchange data analysis. To obtain k_{ex}, a single exponential function was fitted to the time-dependent maximal cross peak intensities of non-overlapping cross peaks:

$$I(t) = I(\infty) + C \cdot \exp(-k_{ex} \cdot t) \qquad \text{(S1)}$$

In equation S1, t is the time between initiation of H/D exchange and the start of an individual HSQC experiment, $I(\infty)$ is the peak intensity at infinite time, C is the pre-exponential factor, and k_{ex} is the amide proton exchange rate. In an analogous manner, when a cross peak (labeled 1) partially overlaps with another one (labeled 2), a sum of two decaying exponentials was fitted to the time-dependent maximal intensity of cross peak-1 to obtain the corresponding k_{ex1}:

$$I(t) = I(\infty) + C1 \cdot \exp(-k_{ex1} \cdot t) + C2 \cdot \exp(-k_{ex2} \cdot t) \qquad \text{(S2)}$$

Some residues show initially that their cross peak intensity increases exponentially as time proceeds and as time progresses further this intensity decreases exponentially. This initial signal increase is attributed to H/D exchange of a relatively rapidly exchangeable proton in the immediate vicinity of the amide proton of interest. As a result, dipole-dipole interactions are affected, causing slower relaxation and concomitant sharpening of the cross peak of the backbone amide of interest. In cases where this phenomenon is observed, equation S2 was fitted to the time-dependent maximal cross peak intensities, using a negative value for parameter $C2$. Constant k_{ex2} is the rate constant for incorporation of a deuteron in immediate vicinity of the amide proton of interest, and k_{ex1} is the rate constant for H/D exchange of the amide proton of interest. Indeed, in most cases, k_{ex2} could be assigned to be due to exchange of an amide proton within hydrogen bonding distance of the amide of interest.

Model for H/D exchange. The time course of exchange can be monitored at the residue level by NMR spectroscopy, since amide protons give rise to a ^{1}H-NMR signal, and replacement of the proton by a deuteron leads to disappearance of this signal.

Quantitative interpretation of H/D exchange is possible using a simple model[38]:

$$Closed(NH) \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} Open(NH) \xrightarrow{k_{int}} Exchanged(ND) \qquad \text{(S3)}$$

In this model, the open or exchange-competent form and the closed or exchange-incompetent form of a protein at the site of a particular amide proton interconvert with rate constants for opening, k_{op}, and closing, k_{cl}. From the open state, exchange takes place with the intrinsic rate constant k_{int}, which depends on amino acid sequence, pD, and temperature and can be calculated using values derived from work on model peptides[40].

Under conditions favoring the closed state (i.e. $k_{op} << k_{cl}$), the observed exchange rate, k_{ex}, equals:

$$k_{ex} = \frac{k_{op} \bullet k_{int}}{k_{cl} + k_{int}} \qquad \text{(S4)}$$

Above pD 4, amide proton exchange is base-catalyzed and k_{int} increases tenfold by every increase of pD by 1 unit. Depending on the ratio of k_{cl} and k_{int}, two limiting cases may be reached (Fig. 4a). If $k_{cl} << k_{int}$ equation S4 reduces to:

$$k_{ex} = k_{op} \qquad \text{(S5)}$$

Under this so-called EX1 condition, k_{ex} of a certain amide proton informs about the rate constant for conversion between the closed and the open state of its micro-environment. If $k_{cl} >> k_{int}$ equation S4 reduces to:

$$k_{ex} = \frac{k_{op} \bullet k_{int}}{k_{cl}} = K_{op} \bullet k_{int} \qquad \text{(S6)}$$

Under this so-called EX2 condition, the ratio of k_{ex} and k_{int} provides the equilibrium constant K_{op} for opening of the protein structure at the backbone amide under investigation. K_{op} is a measure for the corresponding stability ΔG_{op}:

$$\Delta G_{op} = -RT\ln(K_{op}) = -RT\ln\left(k_{op}/k_{cl}\right) = -RT\ln\left(k_{ex}/k_{\text{int}}\right) \qquad \text{(S7)}$$

where R is the gas constant and T the absolute temperature.

To estimate the accuracy with which ΔG_{op} is determined, we mutually compared on a residue basis ΔG_{op}-values of 47 backbone amides of flavodoxin at pD 6.19 and at pD 6.26; these backbone amides exchange according to an EX2 mechanism at both pD-values (see Results section). The average difference in stability (i.e., ΔG_{op}(pD = 6.19) - ΔG_{op}(pD = 6.26)) for these 47 residues equals (0.28 ± 0.35) kcal/mol. This average difference of 0.28 kcal/mol is probably due to slight stabilization of flavodoxin upon lowering pD. The value of 0.35 kcal/mol is a reasonable estimate of the standard deviation of ΔG_{op}.

Determination of rate and equilibrium constants for FMN dissociation from flavodoxin. The dissociation constant of the apoflavodoxin–FMN complex was determined by us[31] using the very strong quenching of FMN fluorescence upon binding of FMN to apoflavodoxin[35,52]. FMN was obtained during the TCA-induced preparation of apoflavodoxin and purified by reverse-phase HPLC. A solution of 1.5 ml containing 188 nM FMN (based on the extinction coefficient at 445 nm of 12.2 $mM^{-1}cm^{-1}$ [53]) in 100 mM KPPi pH 6.0 was titrated with aliquots of 4.4 µM apoflavodoxin in the same buffer. After each addition of protein, the system was allowed to equilibrate for 5 minutes in the dark. Subsequently, FMN fluorescence intensity was determined at 25 °C by using a Cary eclipse fluorimeter (Varian, Palo Alto, CA, USA). Excitation was at 445 nm with a slit of 5 nm; emission was recorded at 525 nm with a slit of 10 nm. The dissociation constant of the apoflavodoxin-FMN complex was determined by fitting the following equation to the observed quenching of fluorescence emission of FMN:

$$F = dF_{end} + F_{\delta} \bullet \left(dC_F - \frac{(C_A + K_D + dC_F) - \sqrt{(C_A + K_D + dC_F)^2 - 4C_A dC_F}}{2} \right) \qquad \text{(S8)}$$

where F is the observed fluorescence intensity after each addition of apoflavodoxin, d the dilution factor (initial volume/total volume), F_{end} the remaining fluorescence intensity after titration (resulting from both fluorescence of flavodoxin and of traces of flavin impurities that are unable to bind to apoflavodoxin), F_{δ} the difference in molar emission intensity between flavodoxin and free FMN, C_F the initial concentration of FMN, C_A the total protein

concentration (i.e. apoflavodoxin + flavodoxin), and K_D the dissociation constant of the apoflavodoxin-FMN complex. We determined apoprotein concentration by measuring absorbance, using an extinction coefficient of 26.500 M^{-1} cm^{-1} at 280 nm. Equation S8 is derived for equimolar binding stoichiometry of FMN to apoflavodoxin. This equation is fitted to the data of Fig. 1a. The total protein concentration (C_A) derived using this fit is identical to the apoprotein concentration as determined by absorption. Consequently, all apoflavodoxin binds FMN.

The rate constant for dissociation of FMN from flavodoxin, k_{off}, was determined by following the increase in fluorescence due to FMN release when flavodoxin was diluted in buffer. Upon dilution the following equilibrium is affected:

$$flavodoxin \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} apoflavodoxin + FMN \qquad \text{(S9)}$$

Return to equilibrium proceeds exponentially as function of time, as it is a first-order process, and the corresponding relaxation time is[37]:

$$1/\tau = k_{on} \cdot ([apoflavodoxin] + [FMN]) + k_{off} \qquad \text{(S10)}$$

where k_{on} is the association rate constant and k_{off} the dissociation rate constant, as shown in equation S9. From this dilution experiment the equilibrium constant for dissociation of FMN from flavodoxin can be retrieved as well, because K_D equals k_{off}/k_{on}.

During dilution experiments, 50, 20, 10 and 5 µl of 362 µM flavodoxin in 100 mM KPPi, pH 5.65, were added to 2000 µl buffer, respectively. As a result, flavodoxin was diluted 41, 101, 201, and 401 times, respectively, and final protein concentrations were 8.8, 3.6, 1.8, and 0.9 µM. Equilibrium of FMN dissociation was reached within 40 minutes. Fluorescence emission of FMN was followed at 525 nm with a slit of 10 nm using a Cary eclipse fluorimeter, while excitation occurred at 450 nm with a slit of 5 nm and sample temperature was set to 25 °C. To determine k_{off}, as well as K_D, the following equation was globally fitted to the four relaxation traces obtained, using ProFit (Quantumsoft, Zürich, Switzerland):

$$F(t) = A \cdot exp(-t/\tau) + F(\infty) + c \qquad \text{(S11)}$$

where F is the observed fluorescence intensity of FMN, A is the amplitude of FMN fluorescence signal change upon dilution of flavodoxin, τ is the relaxation time as described by equation S10 with k_{on} defined as k_{off}/K_D, $F(\infty)$ is the observed fluorescence of FMN after FMN release upon dilution of flavodoxin has reached equilibrium, and c is an adjustable parameter that accounts for offset observed in fluorescence, which is mainly caused by Raman scattering, and differs slightly per relaxation trace, due to among others small differences in cuvettes used. The initial concentration in nM of FMN in the stock of flavodoxin, as well as the final concentration of FMN after cofactor release upon dilution of flavodoxin has reached new equilibrium, is determined in both cases by K_D. Consequently, fluorescence of FMN at the start of the dilution experiment, $F(0)$, and FMN fluorescence upon reaching new equilibrium, $F(\infty)$, can be calculated according to:

$$F(0) = (scaling\ factor) \cdot \frac{-K_D + \sqrt{K_D{}^2 + 4 \cdot K_D \cdot 362 \cdot 10^{-6}}}{2} \cdot d \qquad \text{(S12)}$$

and

$$F(\infty) = (scaling\ factor) \cdot \frac{-K_D + \sqrt{K_D{}^2 + 4 \cdot K_D \cdot 362 \cdot 10^{-6} \cdot d}}{2} \qquad \text{(S13)}$$

with d the dilution factor, i.e., the volume taken out of the stock solution divided by the final volume of the sample, and the scaling factor is $5 \cdot 10^9$, as in our fluorescence emission set-up 1 nM of FMN corresponds to 5 fluorescence units. Hence, amplitude A in equation S11 is:

$$A = F(0) - F(\infty) \qquad \text{(S14)}$$

Backbone amide exchange of apoflavodoxin and flavodoxin occurs according to an EX2 mechanism. To reveal at the residue level how FMN affects the stabilities of flavodoxin's residues against exposure to solvent, we first need to measure H/D exchange rates of backbone amides of the apoprotein (Supplementary Table S1, pD is 6.28). As the residues of apoflavodoxin display EX2 behavior at pD 6.3 [32], these rates are directly converted into ΔG_{op}-values using equation S7. Subsequently, we measure H/D exchange rates of the holoprotein to determine the corresponding ΔG_{op}-values of flavodoxin (Supplementary Table S1, pD is 6.26). At pD 6.3 it is expected that backbone amides of flavodoxin exchange according to an EX2 mechanism, because: (a) flavodoxin is structurally largely identical to

apoflavodoxin[49,34], (b) FMN does not accelerate flavodoxin folding[31], (c) during global unfolding of flavodoxin release of FMN is rate-limiting[31], and (d) stability against global protein unfolding increases considerably upon binding of FMN to apoflavodoxin[31]. When amide protons of flavodoxin exchange according to an EX2 mechanism, their exchange rates directly convert into corresponding ΔG_{op}-values. Consequently, stabilization against exposure to solvent of the residues of apoflavodoxin upon FMN binding is described by (ΔG_{op}(flavodoxin) - ΔG_{op}(apoflavodoxin)) = $\Delta\Delta G_{op}$.

To verify whether exchange of amides of flavodoxin indeed occurs according to an EX2 mechanism at pD 6.26, we measure H/D exchange also at pD 6.19, 7.73 and 8.82. Supplementary Table S2 lists exchange rates for 105 of the 179 backbone amides of flavodoxin.

The following 22 residues show EX2 exchange behavior over the entire pD range investigated (i.e., log(k_{ex}) depends linearly on pD with a slope of about 1): Leu5, Gly8, Arg15, Val17, Lys19, Ile21, Lys22, Leu34, Ile81, Val91, Leu93, Gln99, Gly101, Tyr106, Leu110, Gly111, Lys118, Phe146, Val147, Ala150, Ala169, and Ile171. An example of this EX2 behavior is shown for Leu34 in Fig. 4b. In case of the following six residues log(k_{ex}) depends linearly on pD with a slope ranging in between 1.6 to 1.9: Lys16, Phe77, Val125, Trp128, Val141, and Gln170. Apparently, the corresponding backbone amides, which all reside at flavodoxin's periphery, have stabilities against exposure to solvent that decrease upon increasing pD from 6.19 to 8.82.

In case of Lys13, log(k_{ex}) decreases with increasing pD (Supplementary Figure S4a). The backbone amide proton of Lys13 is hydrogen bonded to the phosphate of FMN (Supplementary Figure S4b). The pK of this phosphate is 5.5 (i.e., mono-anion to di-anion transition)[54]. Deprotonation of this phosphate upon increasing pD explains the exchange behavior observed for Lys13, because this event strengthens the hydrogen bond discussed. However, this strengthening does not significantly reinforce binding between FMN and apoflavodoxin. If FMN binding would become stronger, several other amide protons of flavodoxin should also display a decrease of k_{ex} upon increasing pD, which is not observed.

Five residues (i.e., Thr14, Thr56, Asp98, Tyr102, and Gly148) have log(k_{ex})-values that show a curved dependency on pD. An example of such curved data is shown for Thr56 in Fig. 4b. Clearly, at pD 6.26 this residue exchanges according to an EX2 mechanism, just as is the case for the other four residues mentioned.

Due to overlap of ^{1}H-^{15}N cross peaks, k_{ex} of Phe7, Ala92, Leu96, Asp108, Tyr114, and Leu168 can only be determined at pD 8.82. Similarly, exchange rates of Phe6, Leu50 - Thr54, Phe94, Gly95, Val100, and Trp167 can only be retrieved from HSQC spectra acquired

at pD 6.26 and 8.82. Increase of the exchange rates of Phe6, Leu50 - Gly53, and Phe94 in this pD-range is consistent with exchange moving from an EX2 mechanism at pD 6.26 to an EX1 mechanism at pD 8.82. A partial move towards an EX1 mechanism in this pD regime occurs for Thr54, Gly95, Val100, and Trp167.

Of fifty-five residues k_{ex} could only be determined at pD 6.19 or 6.26 or at both pD-values, but not at higher pD-values, because upon increasing pD exchange becomes so rapid that the corresponding amide protons fully exchange with deuterons within the dead time of the exchange experiment. These residues are: Ile3, Lys23 - Phe25, Thr29 - Ser31, Ala33, Asn37 - Val39, Ala45 - Tyr47, Phe49, Leu57, Gly58, Gly60, Glu61, Gly64, Leu65, Ser73 - Glu76, Lys80, Glu82, Leu84, Lys89, Asn105, Glu112, Leu113, Asp119 - Gly121, Lys123, Ile124, Asp131, Tyr133 - Phe135, Ser137, Glu139, Val142, Asp143, Lys145, Leu151, Leu153, Asp154, Asn155, Arg163, Ala172, Glu174, Phe175, and Leu177. This strong dependence on pD supports that exchange of these residues occurs according to an EX2 mechanism at pD 6.26. In summary, the wealth of exchange data collected support that at pD 6.26 exchange of backbone amides of flavodoxin indeed occurs according to an EX2 mechanism.

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