TABLE S1 Probability of receiver domain K+1 amino acid given K+2 amino acid

Amino at K+2	Abundance at K+2 (%)	% of Wild Type Receiver Domains with Indicated Amino Acid at K+2 that have Pro, Asp, or Ser at K+1		
		Pro	Asp	Ser
Any	100	82	5.3	2.9
Phe	39	99	0.1	0.2
Val	15	91	4.2	1.1
Ile	13	92	3.1	0.6
Leu	5.8	92	1.1	2.7
Tyr	4.7	99	0.1	0.2
Trp	1.7	98	0.4	0.4
Ala	4.9	33	26	12
Ser	3.1	24	17	16
Thr	2.5	23	37	7

Total sample size was 33,252 sequences (1).

TABLE S2 Comparison of *E. coli* CheY autophosphorylation rate constants determined by k_{obs} or $K_{1/2}$ methods

Amino acid at					$k_{phos}/K_{S\ PAM}$		$k_{dephos}/K_{1/2\ PAM}$
D+2	T+1	T+2	K+1	K+2	(M^{-1} s^{-1})	n	(M^{-1} s^{-1})
Asn	Ala	Glu	Pro	Phe	8.1 ± 1	3	8.8 ± 1[a]
Asn	Ala	Glu	Pro	Val	160 ± 6	3	170 ± 20[a]
Asn	Ala	Glu	Pro	Ile	150 ± 0	2	190 ± 30[a]
Asn	Thr	Ser	Pro	Phe	3.5 ± 0.7[b]		3.1 ± 0.9[c]

[a]Value from Table 1.
[b]Value from (2)
[c]Value from Table 3.

TABLE S3 Binding affinity for BeF_3^- of *E. coli* CheY mutants altered at K+2

Amino acid at K+1	Amino acid at K+2	k_{dephos}[a] (min^{-1})	$k_{dephos}/K_{1/2\ PAM}$[a] ($M^{-1}\ s^{-1}$)	$K_d\ BeF_3^-$ (μM)	n
Pro	Phe	3.5 ± 0.3	9.0 ± 1	23 ± 6	4
Pro	Tyr	9.1 ± 0.3	27 ± 2	18 ± 10	3
Pro	Ser	5.8 ± 0.3	44 ± 3	8.7 ± 1	3
Pro	Val	7.6 ± 0.3	170 ± 20	3.6 ± 0.7	3
Pro	Ala	8.7 ± 1	730 ± 90	0.70 ± 0.07	3

[a]Values from Table 2.

TABLE S4 Combined effects of substitutions at K+1 and K+2 on *E. coli* CheY autophosphorylation and autodephosphorylation rate constants[a]

Amino acid at K+1	Amino acid at K+2	Expected Value[b]	Actual Value[c]	Difference[d]
$k_{dephos}/K_{1/2\ PAM}$				
Asp	Ala	3.5	1.4	-2.1
Asp	Thr	1.6	1.3	-0.25
Asp	Val	2.1	1.1	-1.0
Ser	Ala	4.8	1.5	-3.3
k_{dephos}				
Asp	Ala	1.0	0.49	-0.53
Asp	Thr	0.68	0.51	-0.17
Asp	Val	0.89	0.92	0.03
Ser	Ala	1.2	0.80	-0.38

[a]Rate constants from Table 3.

[b]Expected value = ln (rate constant for K+1 mutant/wild-type rate constant) + ln (rate constant for K+2 mutant/wild-type rate constant), in units of -RT.

[c]Actual value = ln (rate constant for K+1/K+2 mutant/wild-type rate constant), in units of -RT.

[d]Difference = Actual value - Expected value, in units of -RT. A difference with an absolute value of less than ln2 = 0.69 suggests no significant interaction (less than a factor of 2) between the kinetic effects of substitutions at K+1 and K+2, i.e. the effects are additive. A difference < -ln2 indicates antagonism and a difference > ln2 indicates synergy.

TABLE S5 Combined effects of substitutions at D+2/T+1/T+2 and K+2 on *E. coli* CheY autophosphorylation and autodephosphorylation rate constants[a]

Amino acid at D+2	Amino acid at T+1	Amino acid at T+2	Amino acid at K+2	Expected Value[b]	Actual Value[c]	Difference[d]
$k_{dephos}/K_{1/2\ PAM}$						
Glu	Ser	Val	Ser	2.6	2.7	0.061
Glu	Ser	Leu	Gln	4.0	4.0	-0.029
Gln	Ala	Asn	Val	4.3	4.0	-0.37
Asn	Thr	Ser	Val	1.9	1.1	-0.77
Glu	Ser	Arg	Tyr	4.1	3.4	-0.70
k_{dephos}						
Glu	Ser	Val	Ser	1.8	1.3	-0.51
Glu	Ser	Leu	Gln	2.0	1.8	-0.23
Gln	Ala	Asn	Val	0.22	-0.090	-0.31
Asn	Thr	Ser	Val	0.72	-0.090	-0.81
Glu	Ser	Arg	Tyr	1.5	1.0	-0.52

[a]Rate constants from Table 3.

[b]Expected value = ln (rate constant for D+2/T+1/T+2 mutant/wild-type rate constant) + ln (rate constant for K+2 mutant/wild-type rate constant), in units of -RT.

[c]Actual value = ln (rate constant for D+2/T+1/T+2/K+2 mutant/wild-type rate constant), in units of -RT.

[d]Difference = Actual value - Expected value, in units of -RT. A difference with an absolute value of less than ln2 = 0.69 suggests no significant interaction (less than a factor of 2) between the kinetic effects of substitutions at D+2/T+1/T+2 and K+2, i.e. the effects are additive. A difference < -ln2 indicates antagonism and a difference > ln2 indicates synergy.

TABLE S6 Frequency of amino acids at K+1 and K+2 by response regulator family[a]

Amino acids at		Frequency of indicated amino acid in wild-type receiver domains (%)[b]							
K+1	K+2	All receivers	OmpR/ PhoB	FixJ/ NarL	NtrC	LytR	Single domain	Hybrid kinases	Other
Pro	Phe	40	84	7.8	48	21	38	27	26
Pro	Ile/Leu/Val[c]	29	7.2	5.3	32	68	36	49	34
Pro	Trp/Tyr	6.2	4.9	0.5	11	6.1	5.8	9.1	6.9
Pro	Ala	1.6	0.6	0.2	1.3	0.9	2.5	1.6	3.2
Pro	Cys/Gly/Ser/Thr	2.6	1.6	0.3	3.1	0.4	4.1	1.2	6.7
Pro	Asn/Gln/Met	0.7	0.0	0.0	0.2	0.1	1.5	0.7	1.1
Pro	Arg/Asp/Glu/His/Lys	0.9	0.0	0.0	0.1	0.0	0.8	0.1	4.7
Pro	Pro	0.5	0.1	0.1	0.7	0.2	1.0	0.2	1.3
Pro	Any	82	99	14	96	96	90	89	84
Asp	Any	5.3	0.3	35	0.3	0.9	1.5	1.4	2.2
Ser	Any	2.9	0.3	11	0.4	0.6	3.3	2.1	1.8
Gly	Any	2.4	0.2	5.8	1.8	0.3	1.3	3.8	2.7

[a]Response regulator families from (1). Sample sizes were: All receivers, 33,252; OmpR/PhoB, 6,697; FixJ/NarL, 4,081; NtrC, 1,920; LytR, 1,383; Single domain, 5,905; Hybrid kinase, 8,113; Other, 5,345.

[b]Values <1% rounded to nearest 0.1%.

[c]The groupings of K+2 amino acids follow the clusters of rate constants indicated in Figure 2. We have no kinetic data for the effects of Cys, His, or Pro at K+2. Based on the clustering of K+2 amino acid abundance by side chain chemical properties, here Cys is grouped with Ser/Thr and His is grouped with charged amino acids.

REFERENCES

1. Page SC, Immormino RM, Miller TH, Bourret RB. 2016. Experimental analysis of functional variation within protein families: Receiver domain autodephosphorylation kinetics. J Bacteriol 198**:**2483-2493. https://doi.org/10.1128/JB.00853-15
2. Immormino RM, Silversmith RE, Bourret RB. 2016. A variable active site residue influences the kinetics of response regulator phosphorylation and dephosphorylation. Biochemistry 55**:**5595-5609. https://doi.org/10.1021/acs.biochem.6b00645