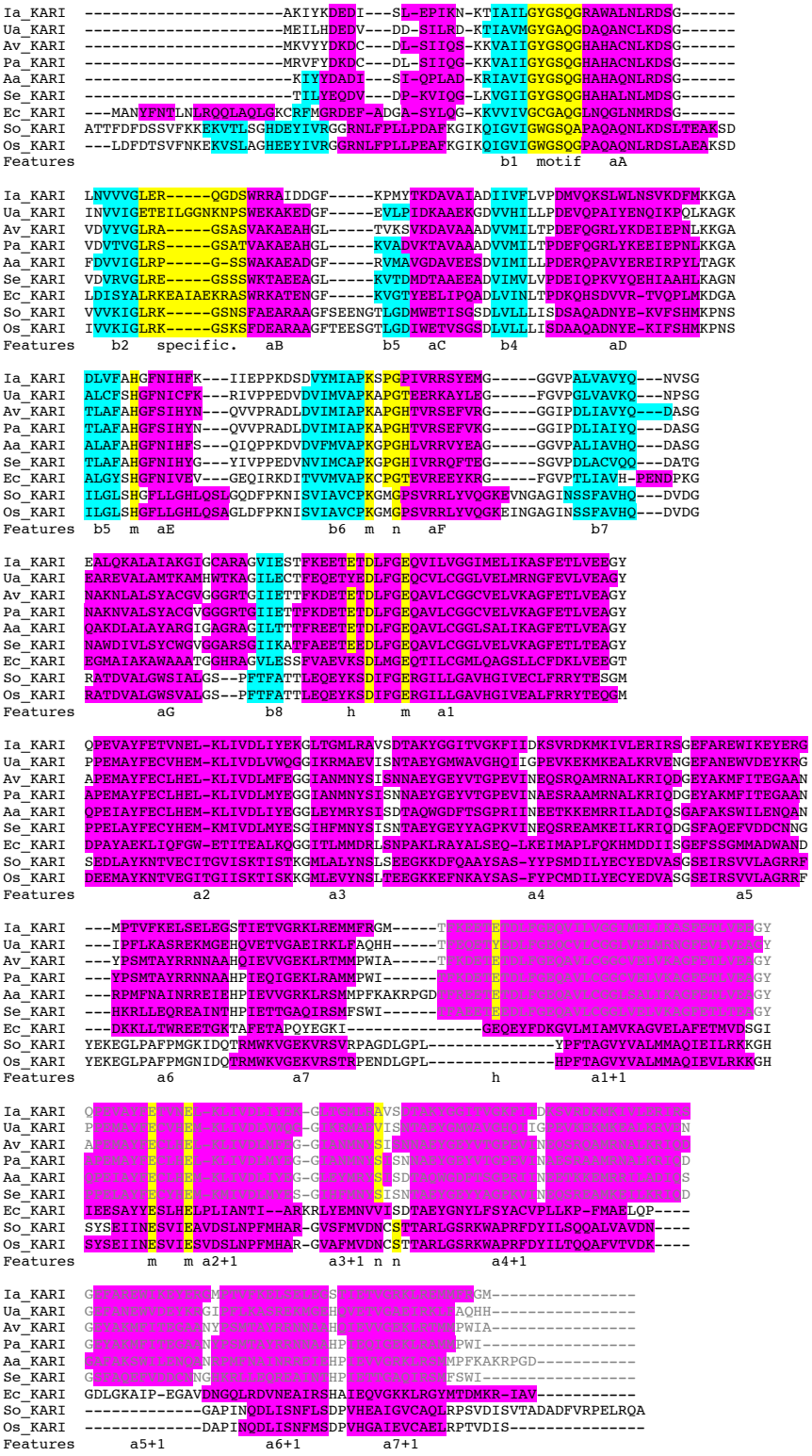
Supplemental Information 1: Specificity loop length and KARI class of 3,422 unique KARIs retrieved from Pfam. Four additional Class I KARIs had other specificity loop lengths. Green indicates enzymes with representative structures published prior to this study.

Loop Length	**6**	**7**	**12**
Class I	595	2260	5
Class II	0	20	538

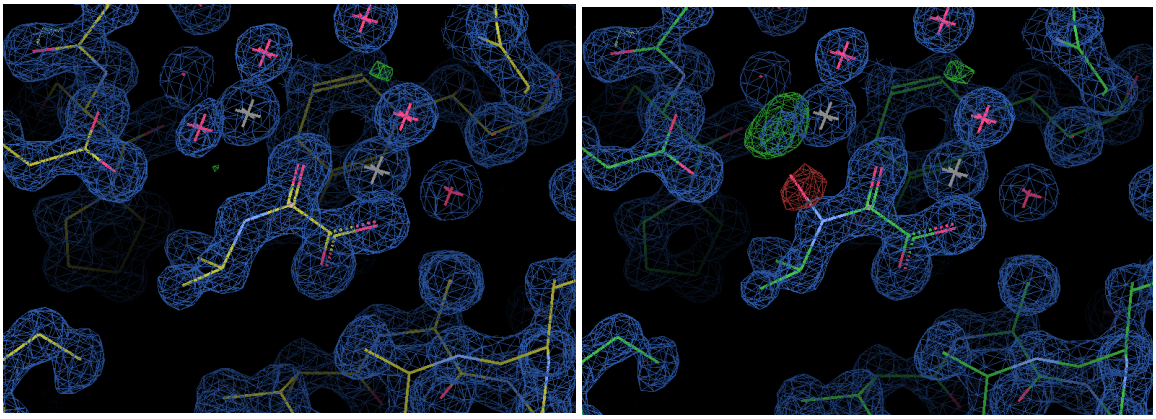
Supplemental Information 2: Refinement Statistics for structures presented in this paper. Values in parentheses represent statistics from the highest-resolution shell.

	Aa_KARI_holo 4TSK	**Ua_KARI_holo 4XDY**	**Ia_KARI_holo 4XDZ**	**Ia_KARI_apo 4XEH**	**Av_KARI_met 4XIY**
			Data Collection		
Space Group	*P* 2 3	*P* 2_1 2_1 2	*P* 2_1	*P* 2_1 2_1 2	*P* 2_1 3
Cell Dimensions					
***a*,*b*,*c* (Å)**	124.10, 124.10, 124.10	141.75, 148.60, 49.70	54.58, 90.75, 69.53	67.48, 112.35, 46.07	185.05, 185.05, 185.05
***α*,*β*,*γ* (°)**	90, 90, 90	90, 90, 90	90, 100.3, 90	90, 90, 90	90, 90, 90
Resolution (Å)	124.10-2.50 (2.57-2.50)	102.57-1.54 (1.58-1.54)	68.42-1.15 (1.21-1.15)	57.85-1.39 (1.43-1.39)	82.76-2.50 (2.64-2.50)
$R_{p.i.m}$ (%)	5.3 (61.8)	4.4 (49.8)	5.4 (64.3)	2.2 (107.9)	5.7 (31.3)
Mn(I)/sd	8.9 (0.8)	11.1 (1.5)	6.9 (0.9)	13.6 (0.6)	10.9 (2.6)
Completeness (%)	97.7 (89.1)	97.3 (71.8)	95.0 (82.4)	96.8 (76.0)	99.5 (99.9)
Redundancy	3.4 (2.9)	4.5 (4.5)	3.0 (2.5)	3.6 (3.9)	3.5 (3.6)
			Refinement		
No. reflections	22,066	145,320	224,341	65,375	72,509
***R_{work}*/*R_{free}* (%)**	15.6/18.2 (21.2/27.2)	17.9/20.9 (39.1/38.4)	15.8/19.0 (33.2/34.1)	18.0/23.5 (38.2/38.0)	19.4/24.4 (28.7/32.1)
No. atoms					
Protein	2,585	5,296	5,139	2,561	10,017
Ligand/ion	61	126	178	0	48
Water	46	359	595	101	40
RMSD					
Bond lengths (Å)	0.018	0.023	0.024	0.019	0.015
Bond angles (°)	1.998	2.038	2.461	1.930	1.719
Ramachandran map analysis					
Favored	313	653	589	309	1,194
Allowed	17	20	29	13	99
Outliers	1	0	0	0	23

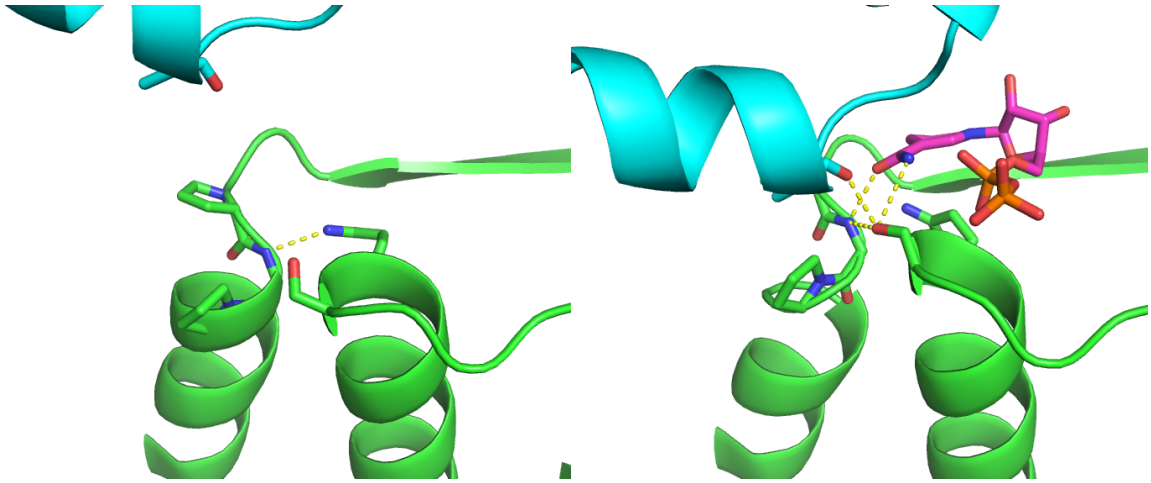
Supplemental Information 3: Sequence alignment of crystallized KARIs. Helices are colored in magenta, and β-strands in cyan. Yellow indicates special features, labeled with the following abbreviations: motif = diphosphate binding motif; specific. = specificity loop; m = metal-binding residue; n = nicotinamide amide-binding residue; h = hinge point (when unambiguously definable). Metal-binding and nicotinamide amide-binding residues are shown only for one active site in the Class I proteins. The knotted domain of the Class I KARIs has been duplicated for clarity; duplicate residues are shown with grey text.


Ia_KARI
Ua_KARI
Av_KARI
Pa_KARI
Aa_KARI
Se_KARI
Ec_KARI
So_KARI
Os_KARI
Features

Supplemental Information 4: Electron density around the substrate of Ia_KARI_holo indicates dehydroxylation of IpoHA. Left: final structure, modelled with *N*-isopropyloxamate (ligand 40E). Right: modelled instead with *N*-hydroxy-*N*-isopropyloxamate (IpoHA; ligand HIO). The F_o-F_c map (red and green) clearly shows the absence of the *N*-hydroxy moiety. ($2F_o$-F_c map contoured to 1.5σ, F_o-F_c map contoured to 4.0σ).



Supplemental Information 5: Structural rearrangements around NADPH nicotinamide in Ia_KARI. Binding of the nicotinamide of NADPH (magenta) to the the αA-helix and the β6αF-loop recruits the α3-helix of the other chain of the dimer (cyan).



Ia_KARI_apo Ia_KARI_holo

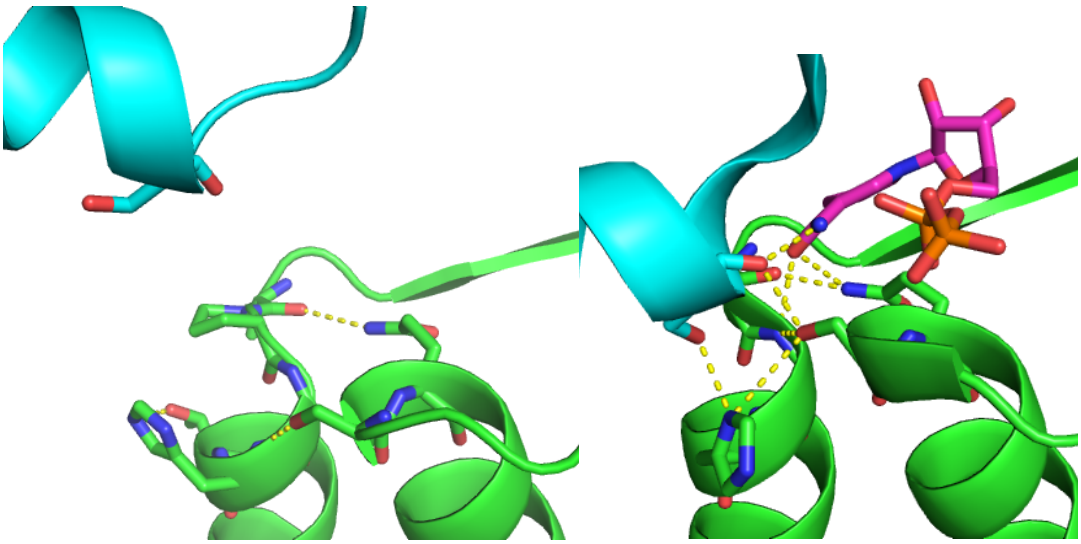
Supplemental Information 6: % Identity between mesophilic Class I KARIs as determined by Clustal Omega.

%ID between Av_KARI and:	
Ia_KARI	46.9%
Pa_KARI	93.2%
Se_KARI	55.9%

Supplemental Information 7: Amino acid identities and numberings in mesophilic Class I KARIs

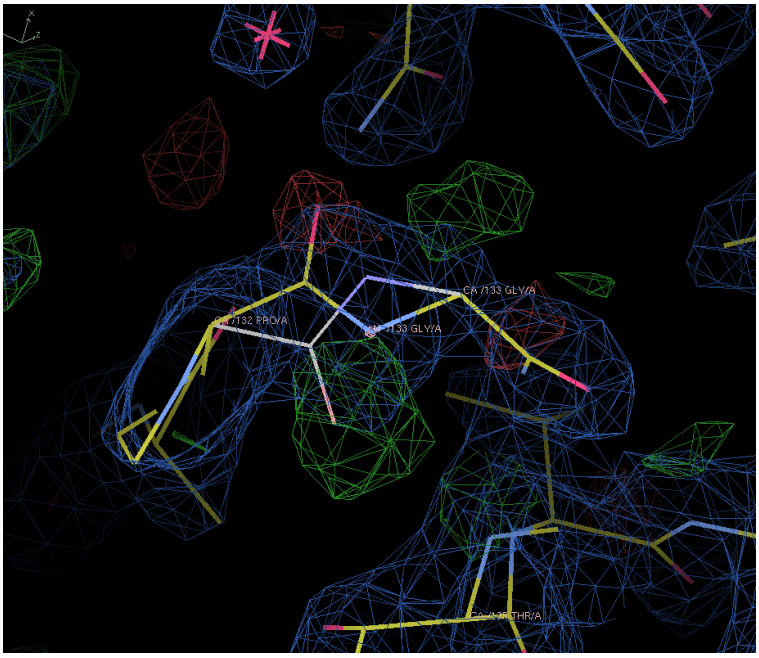
Position in Pa_KARI	Position in Av_KARI	Position in Se_KARI
H107	H107	H118
K130	K130	K141
D190	D190	S201
E194	E194	E205
E226	E226	E237
E230	E230	E241
S249	S249	S260
H134	H134	H145
S26	S26	S37
P132	P132	P143
G133	G133	G144
A131	A131	G142

Supplemental Information 8: Structural rearrangements around NADPH nicotinamide in Av_KARI/Se_KARI. As with Ia_KARI, binding of the nicotinamide of NADPH (magenta) to the the αA-helix and the β6αF-loop recruits the α3-helix of the other chain of the dimer (cyan); in these KARIs more hydrogen bonds are present and undergo rearrangements of the hydrogen bond network.

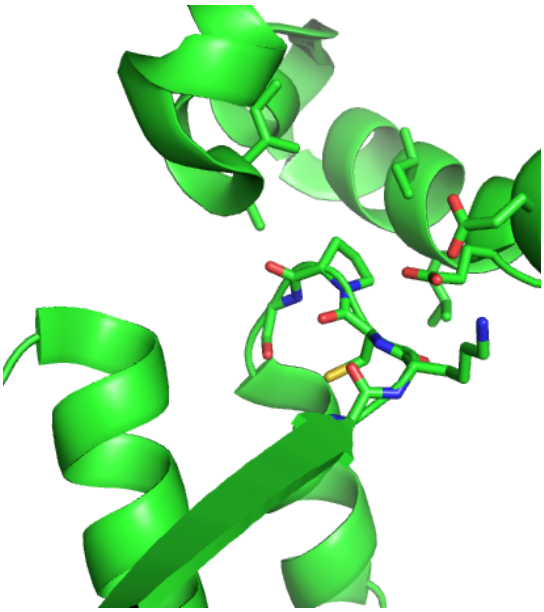


Av_KARI_met Se_KARI_holo

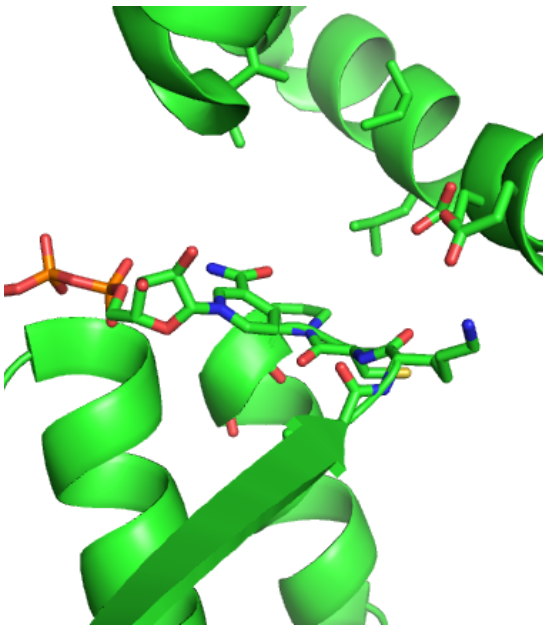
Supplemental Information 9: Modelling error at P132-G133 in Pa_KARI_apo. The F_o-F_c omit map (red and green) shows differential density around the peptide bond in the published structure (yellow) that is resolved by implementing a 180° peptide flip (white). This alteration is corroborated by improved backbone hydrogen bond interactions. ($2F_o$-F_c map contoured to 1.5σ, F_o-F_c map contoured to 2.9σ).



Supplemental Information 10: Structural rearrangements around NADPH nicotinamide in Ec_KARI



Ec_KARI_apo



Ec_KARI_holo