Supporting Information for:

"The epoxyqueuosine reductase QueH in the biosynthetic pathway to tRNA queuosine is a unique metalloenzyme"

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Supplementary Figures/Tables

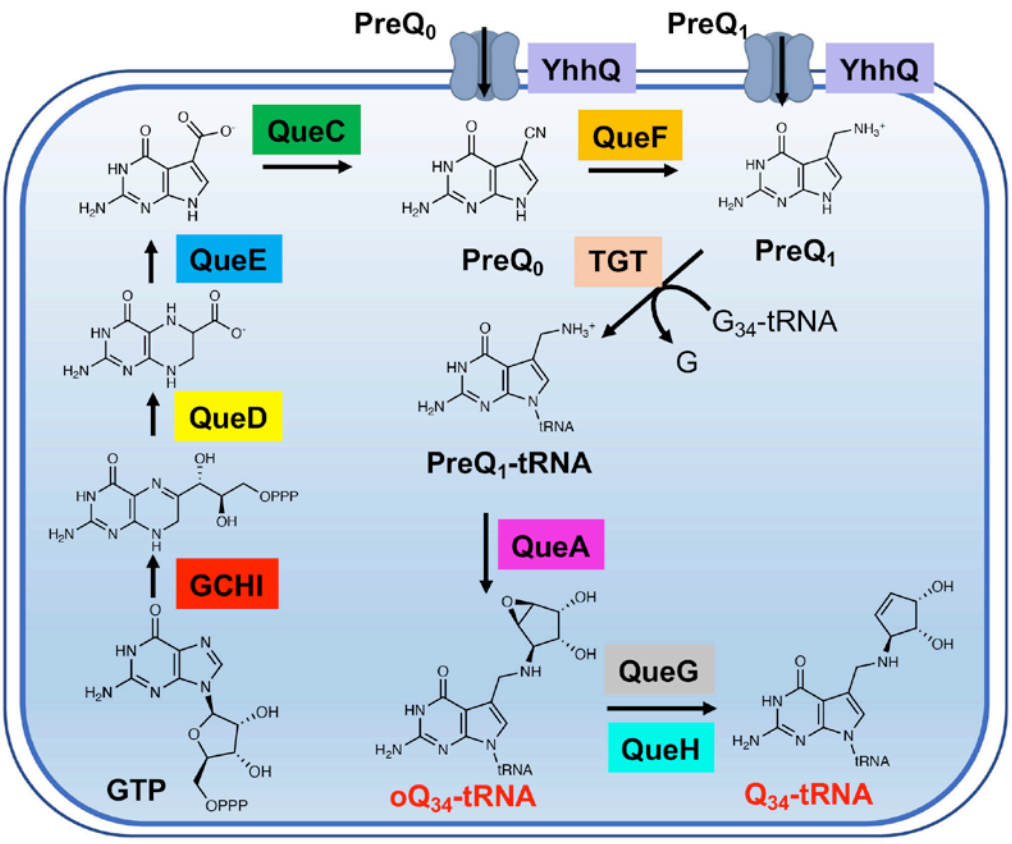
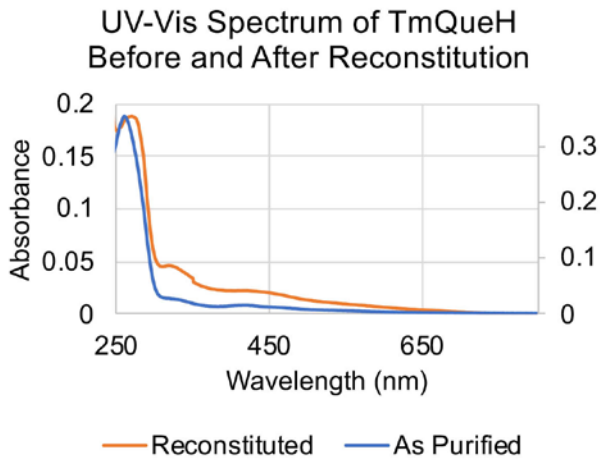


Figure S1. Summary of the queuosine biosynthetic pathway.



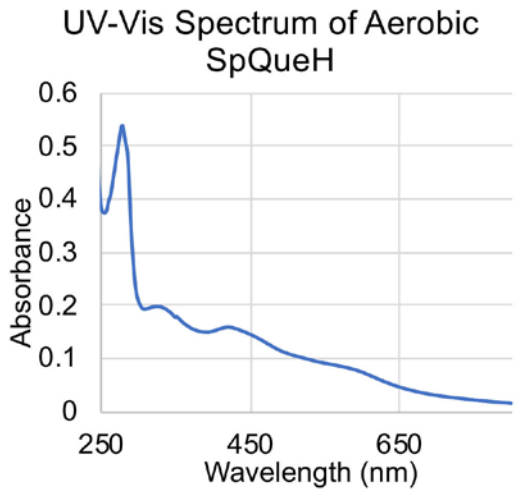


Figure S2. UV-Vis spectra of TmQueH before (blue) and after (red) reconstitution. UV-vis spectra of aerobically purified SpQueH.

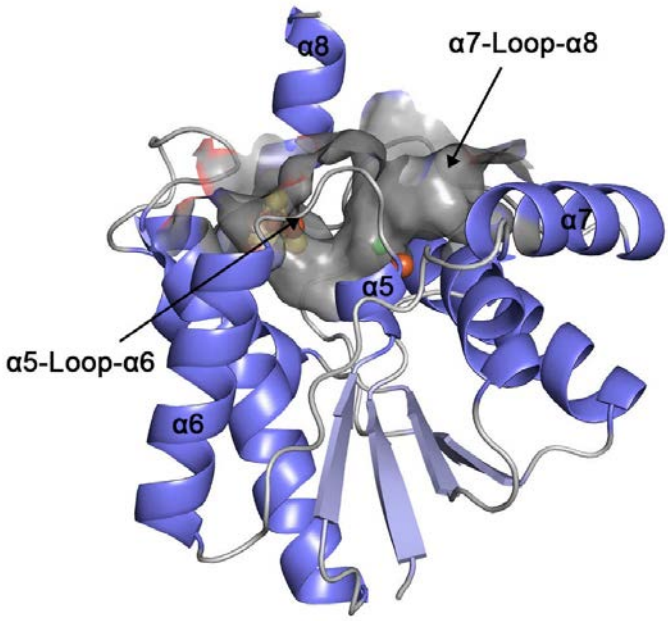


Figure S3. Graphical representation of the QueH substrate binding pocket located between two concurrent protein loops.

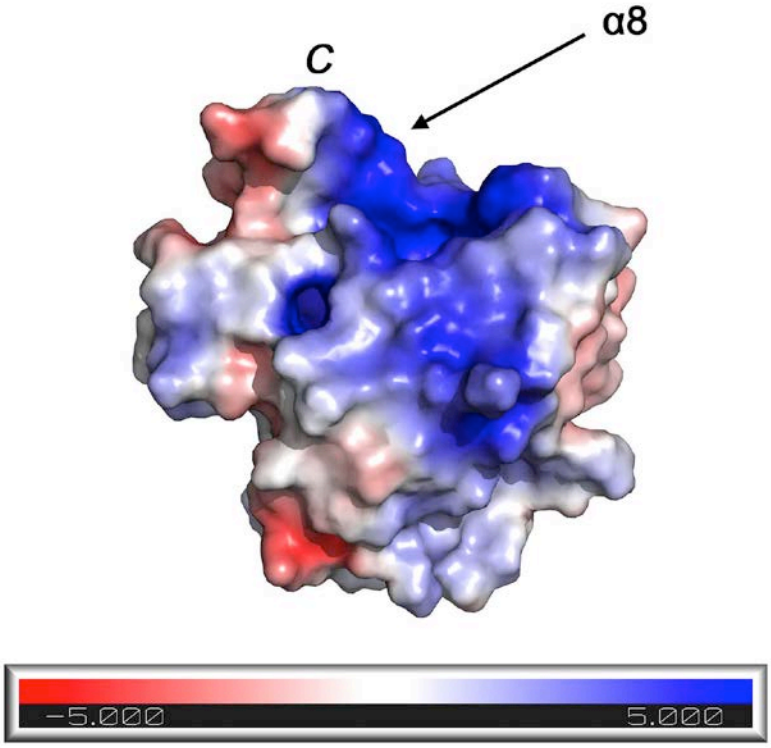
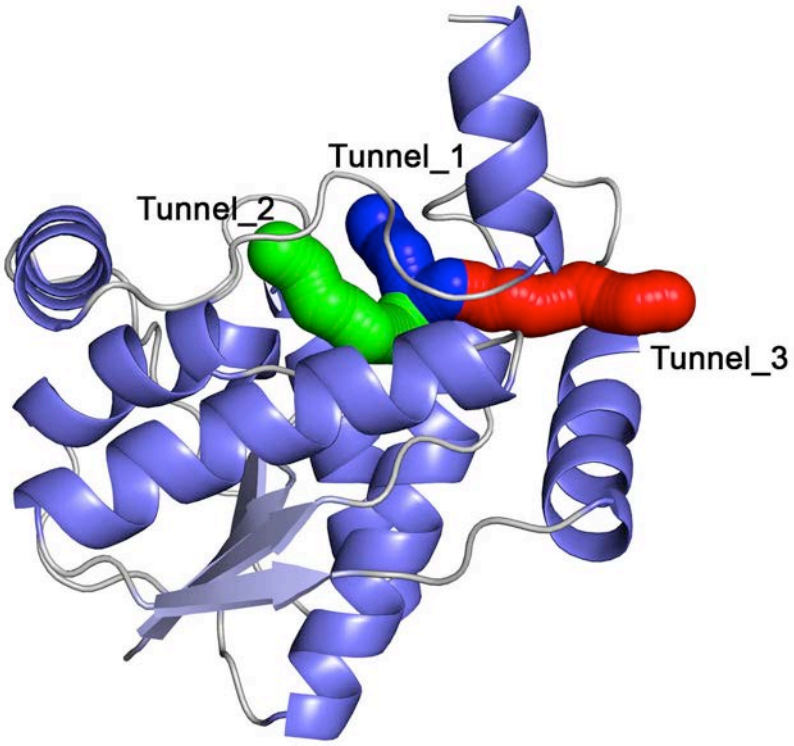


Figure S4. Calculated electrostatic surface map of QueH



ID	Avg_BR [Å]	Max_BR [Å]	Avg_L [Å]
Tunnel_1	1.594	1.59	8.881
Tunnel_2	1.934	1.93	15.547
Tunnel_3	0.977	0.98	14.043

Figure S5. Three solvent accessible tunnels, calculated by CAVER, leading between the [4Fe-4S] cluster active site and surrounding solvent.

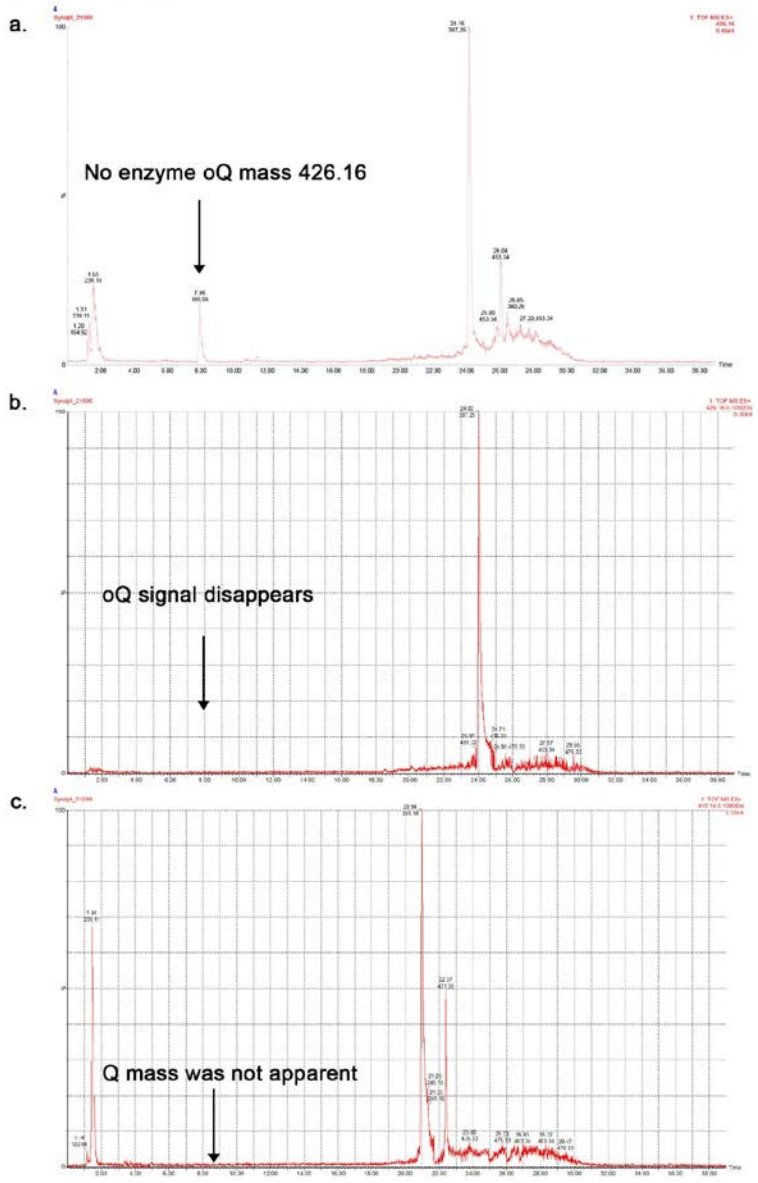


Figure S6. LC-MS analysis of a recombinant TmQueH *in vitro* reaction. **a.** Mass spectroscopy of oQ in the absence of TmQueH at 7.96 min. **b.** Disappearance of oQ signal after treating with 1 μM TmQueH. **c.** No Q signal at 8.60 min in the presence of 1 μM TmQueH.

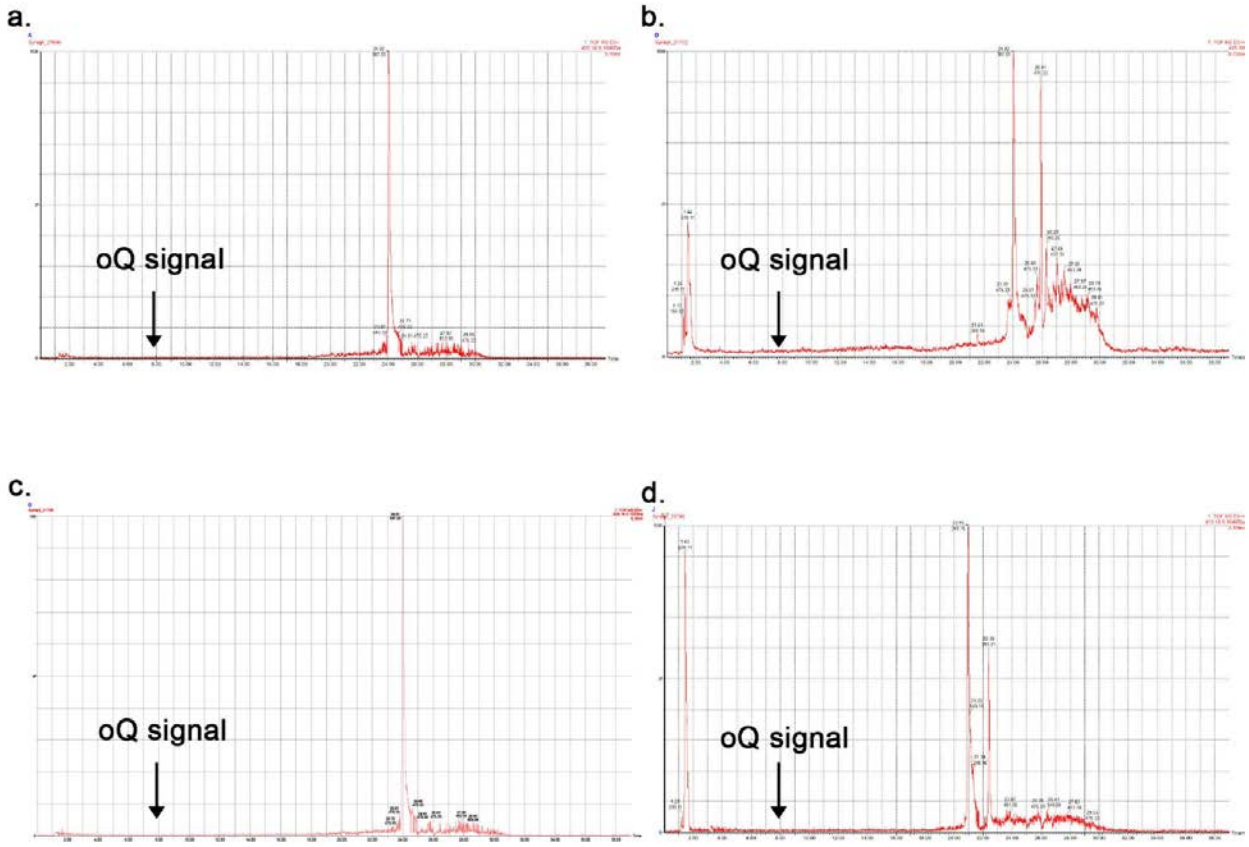


Figure S7 | Mass spectrometry-based TmQueH *in vitro* activity. **a.** Mass spectroscopy of oQ disappearance in the presence of 1 μM untagged TmQueH (Reaction A in Supplementary Table 2). **b.** Mass spectroscopy of oQ disappearance in the presence of 10 μM untagged TmQueH (Reaction D in Supplementary Table 2). **c.** Mass spectroscopy of oQ disappearance in the presence of 1 μM tagged TmQueH (Reaction G in Supplementary Table 2). **d.** Mass spectroscopy of oQ disappearance in the presence of 10 μM tagged TmQueH (Reaction J in Supplementary Table 2).

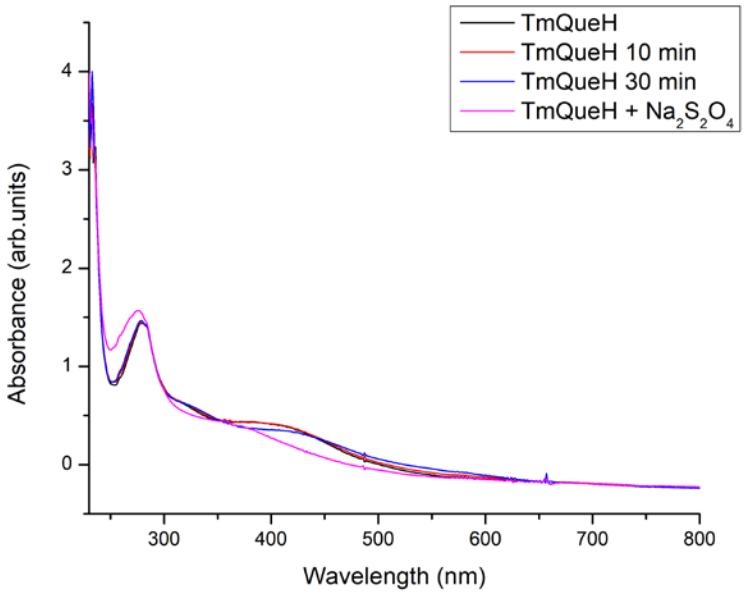


Figure S8 | UV-vis spectrometry of TmQueH. Black: anaerobically prepared sample, pink: sample reduced with sodium dithionate, red and blue:QueH after 10- and 30-min exposure to air, respectively.

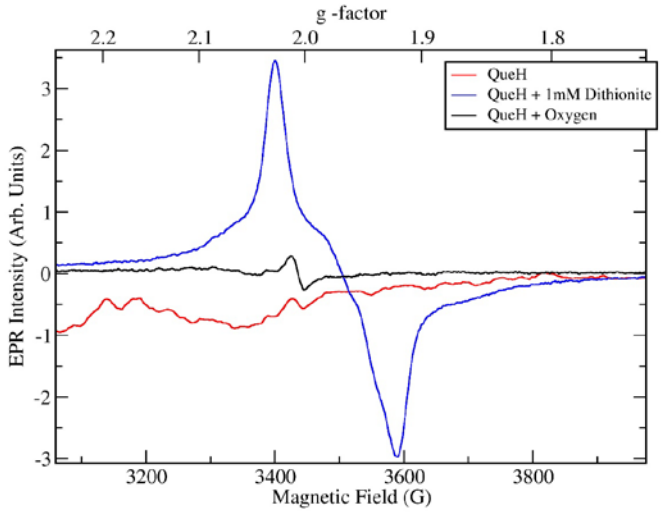


Figure S9 | EPR spectra from fig. 3C expanded into a narrow field range of approximately +/- 400 G around g = 2. Acquisition parameters are given in Materials and Methods.

No:	PDB	Z	rmsd	lali	nres	%id
1	4kr6	11.2	2.7	153	388	10
2	5gha	10.9	3.7	150	310	11
3	6scy	10.8	3.1	140	297	11
4	2deu	10.8	3.3	158	364	9
5	1kor	10.7	3.5	154	387	8
6	3bl5	10.5	3.4	141	195	9

Table S1 | Structural homology, DALI server, search results

No Tag Reaction (1 μM)					
	A	B	C	Final Concentration	Units
	All	No Enzyme	No tRNA		
50 mM HEPES pH 8.0	48 μL	48 μL	48 μL	24	mM
tRNA (5.4 μg/ μL)	40 μL	40 μL	0 μL	200	μg/ 100 μL
50 μM TmQueH	2 μL	0 μL	2 μL	1	μM
1 mM Sodium Dithionite	10 μL	10 μL	10 μL	100	μM
Storage Buffer	0 μL	2 μL	0 μL	N/A	N/A
ddH_2O	0 μL	0 μL	40 μL	N/A	N/A

No Tag Reaction (10 μM)					
	D	E	F	Final Concentration	Units
	All	No Enzyme	No tRNA		
50 mM HEPES pH 8.0	45.8 μL	45.8 μL	45.8 μL	22.9	mM
tRNA (5.4 μg/ μL)	40 μL	40 μL	0 μL	200	μg/ 100 μL
240 μM TmQueH	4.2 μL	0 μL	4.2 μL	10.08	μM
1 mM Sodium Dithionite	10 μL	10 μL	10 μL	100	μM
Storage Buffer	0 μL	4.2 μL	0 μL	N/A	N/A
ddH_2O	0 μL	0 μL	40 μL	N/A	N/A

Tag Reaction (1 μM)					
	G	H	I	Final Concentration	Units
	All	No Enzyme	No tRNA		
50 mM HEPES pH 8.0	48 μL	48 μL	48 μL	24	mM
tRNA (5.4 μg/ μL)	40 μL	40 μL	0 μL	200	μg/ 100 μL
50 μM TmQueH	2 μL	0 μL	2 μL	1	μM
1 mM Sodium Dithionite	10 μL	10 μL	10 μL	100	μM
Storage Buffer	0 μL	2 μL	0 μL	N/A	N/A
ddH_2O	0 μL	0 μL	40 μL	N/A	N/A

Tag Reaction (10 μM)					
	J	K	L	Final Concentration	Units
	All	No Enzyme	No tRNA		
50 mM HEPES pH 8.0	48.1 μL	48.1 μL	48.1 μL	24.05	mM
tRNA (5.4 μg/ μL)	40 μL	40 μL	0 μL	200	μg/ 100 μL
540 μM TmQueH	1.9 μL	0 μL	1.9 μL	10.26	μM
1 mM Sodium Dithionite	10 μL	10 μL	10 μL	100	μM
Storage Buffer	0 μL	1.9 μL	0 μL	N/A	N/A
ddH_2O	0 μL	0 μL	40 μL	N/A	N/A

Reagent	Final Concentrations
Benzonase	0.2 U/ μL
Phosphodiesterase I	0.002 U/ μL
Alkaline Phosphatase	0.02 U/ μL
QueH Reaction (2 μg/ μL tRNA)	20 μg tRNA

Table S2 | Recombinant QueH *in vitro* reaction conditions