Supporting information:

One substrate, five products: Reactions catalyzed by the Dihydroneopterin Aldolase from *Mycobacterium tuberculosis*

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Figure S1: DHNA analysis by LC-ESI-MS

Samples of DHNA were diluted with 2% acetonitrile/0.1% TFA to obtain a final concentration of 1 μM. The injector with a 50 μl loop of a Hewlett-Packard HP1100 HPLC was connected to a C-18 PepMap μ-Pre-column trap (C18 PepMap, 100 A, 1mm x 15 mm, Dionex) which was directly connected to a Rheodyne Divert/Inject valve on the Thermo Electron Finnigan LTQ mass spectrometer. For each analysis, 10 pmol (10 μl) of the sample or blank was injected. The following gradient for solvent A (5% acetonitrile/0.1% formic acid) and solvent B (95% acetonitrile/0.1% formic acid) at a flow rate of 100 μl/min was used: 0-5 min, 2% B; 5-7 min, 50% B; 7-12 min, 90%B; 12-17 min, 2%B. LC-ESI-MS data were acquired from 5 to 12 min. Blank samples were injected in between sample injections. The electrospray voltage used was 4kV. Ten pmol (10 μl) of 1 μM Myoglobin standard (16,951.5 Da) was used as a reference or control to compare the accuracy of the molecular weights of the samples injected. The protein molecular weights were deconvoluted using MagTran and ProMass.

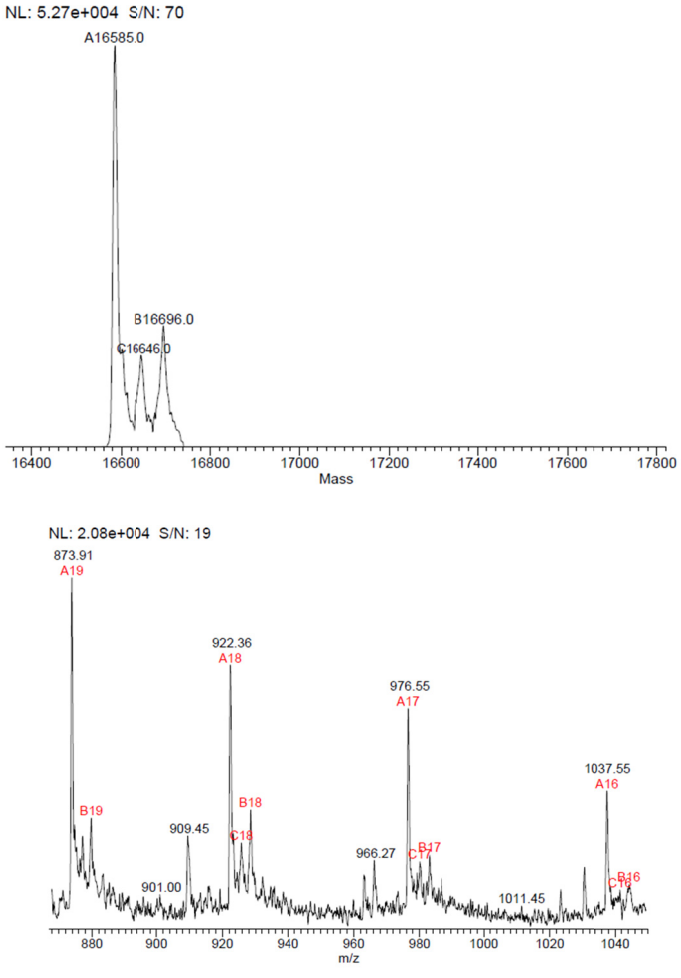
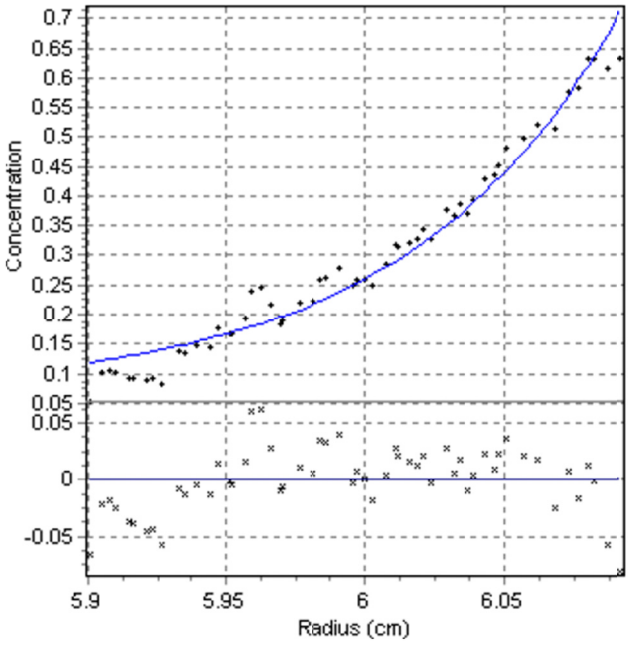
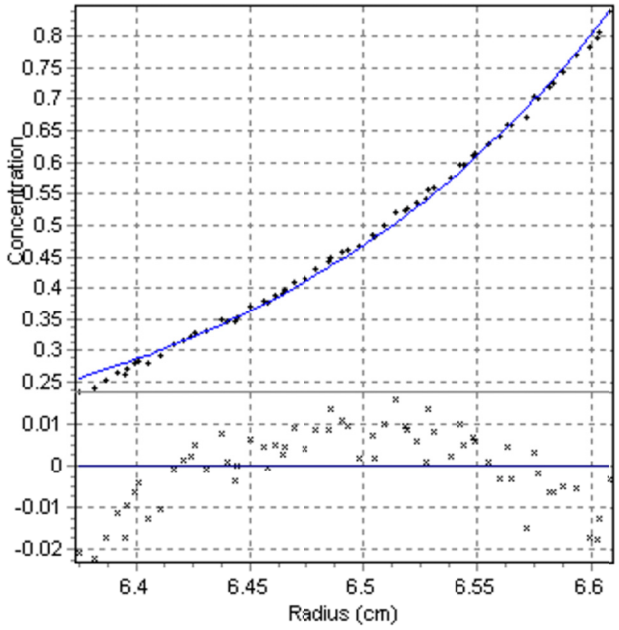


Figure S2: Sedimentation equilibrium ultracentrifugation

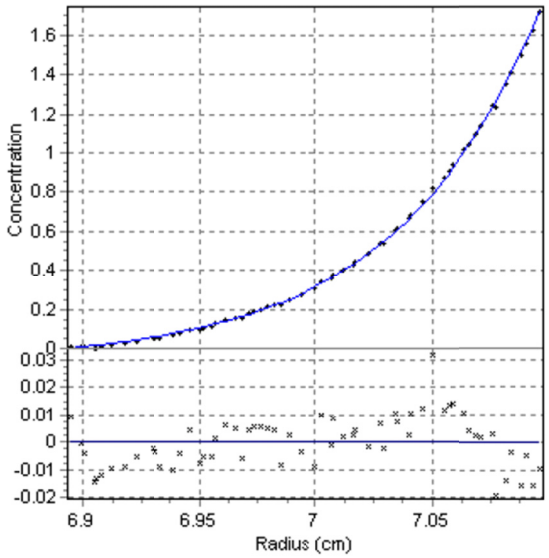
A – free DHNA, 25μM. Fit fives MW = 62,299 ± 25,715 Da.



B - 25μM DHNA + 25μM NP (DHNP analog) Fit gives MW = 117,853 ± 9,774 Da



C - 25μM DHNA + 25μM DHXP
Fit gives MW = 127,525 ± 4,105 Da



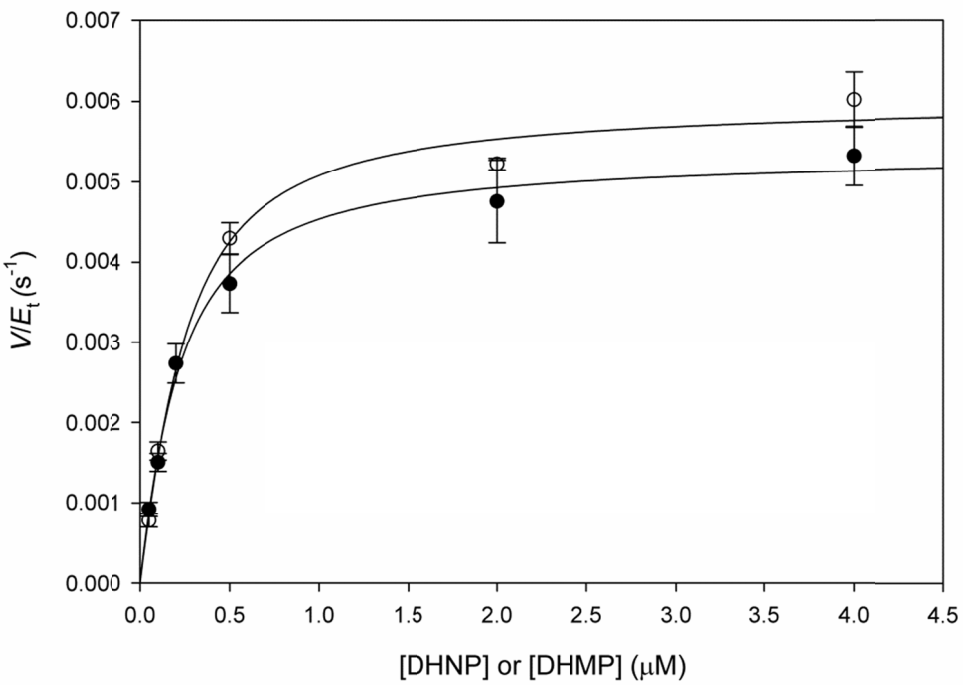


Figure S3: Comparison between DHNP and DHMP as substrates. Fluorescence-based assay comparing DHNP and DHMP as substrates. HP formation was monitored with an excitation wavelength of 430 nm, followed by an increase in fluorescence emission at 535 nm due to HP formation. The slits were 2 nm and 10 nm for excitation and emission, respectively. Reaction with 100 nM *Mt*DHNA, and varying concentrations of DHNP (●) or DHMP (○) in 100 mM HEPES pH 7.0 with 50 mM NaCl, 1mM EDTA, 5 mM DTT, 1% BSA. Temperature was equilibrated for 2 minutes, and reactions were monitored for 3-5 minutes. The first 30 seconds of the reaction with DHNP were excluded since there was a lag period. Data were fitted to eq. S1, giving K_{DHNP} = 165 ± 26 nM, K_{DHMP} = 154 ± 27 nM, $k_{cat\text{-}DHNP}$ = 0.0060 ± 0.0001 s^{-1}, $k_{cat\text{-}DHMP}$ = 0.0070 ± 0.0001 s^{-1}

$$v = V_{max}. \frac{(E_0+S_T+K_m) - \sqrt{(E_0+ S_T+ K_m)^2 - 4.E_0.S_T}}{2.E_0} \quad \text{(S1)}$$

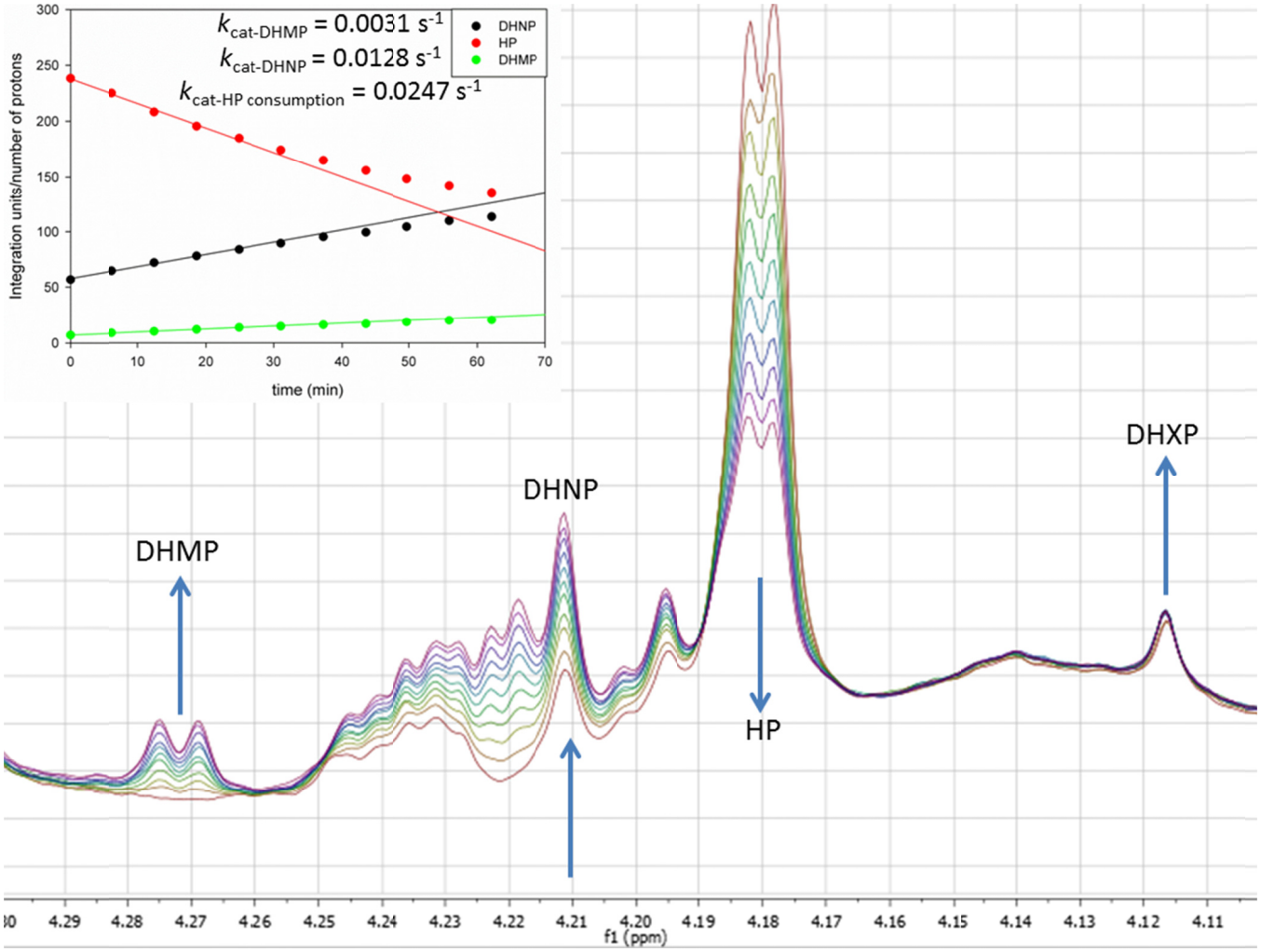


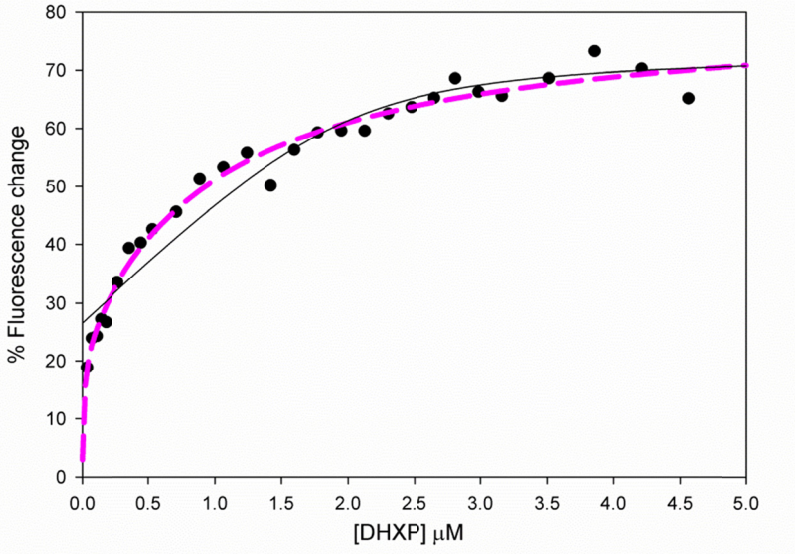
Figure S4: Reverse reaction by NMR-based assays. Reaction with 1mM HP, 50 mM GA, 3 μM in 100 mM Phosphate pH 7.0 with 50 mM NaCl, 1mM EDTA, 5 mM DTT, 1% BSA. Reactions were monitored at room temperature, each trace is the average of 64 scans, taken every 6 minutes. Data for rate of formation of DHXP could not be fitted due to background issues.

Figure S5: DHXP Equilibrium binding

Figure S4: Continuous line: fit to a one site quadratic equation (eq. S2). K_{d1}= 0.17 ± 0.15; dashed line: fit to a two site binding hyperbolic equation.

K_{d1} = 0.01 ± 0.01

K_{d2} = 0.98 ± 0.24



$$F = \frac{(E_0+S_T+K_d) - \sqrt{(E_0+S_T+K_d)^2 - 4.E_0.S_T}}{2.E_0} \quad \text{eq. S2}$$

Figure S6: Coupled assay with formate dehydrogenase. In this assay, 100 μM HP was used as substrate for *Mt*DHNA, and 10 μM formate dehydrogenase from *Candida boidinii* with 1 mM NAD^+ were added to the reaction mixture. The total amount of NADH formed was calculated based on the total change in absorbance at 340 nm using ε_{340} = 6,220 M^{-1} cm^{-1}. The curve in black was collected before adding *Mt*DHNA, the curve in red is after the addition of 10 μM *Mt*DHNA.

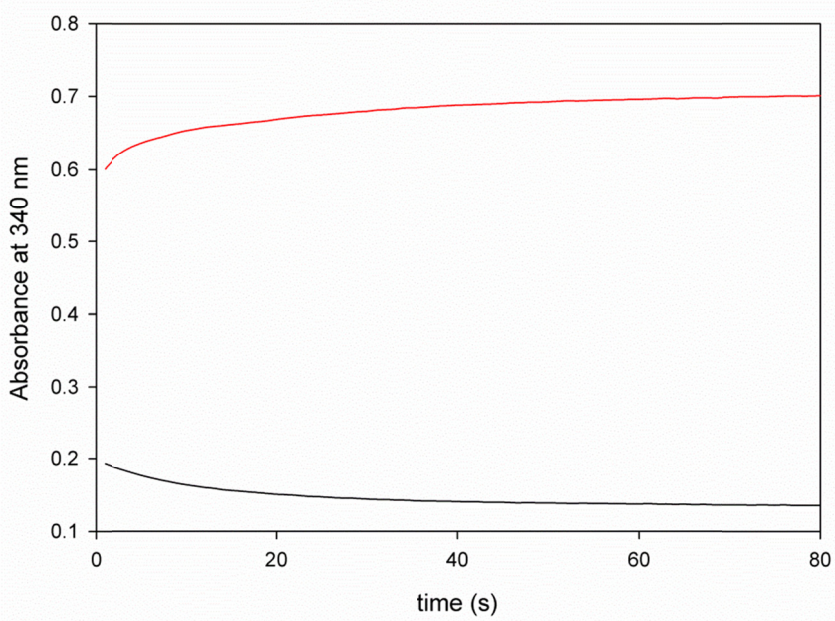
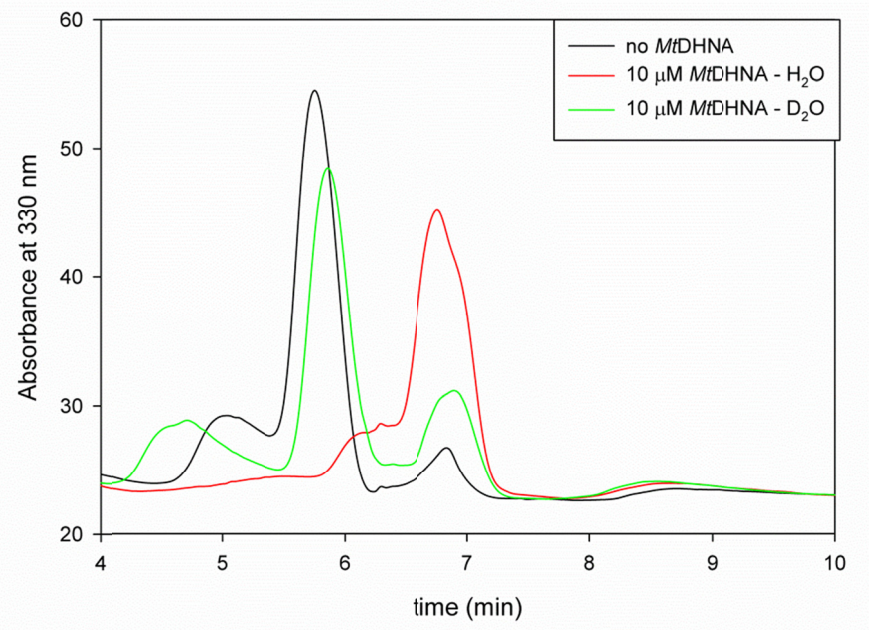


Figure S7: HPLC analysis of end-point reactions under single-turnover conditions in H_2O and D_2O

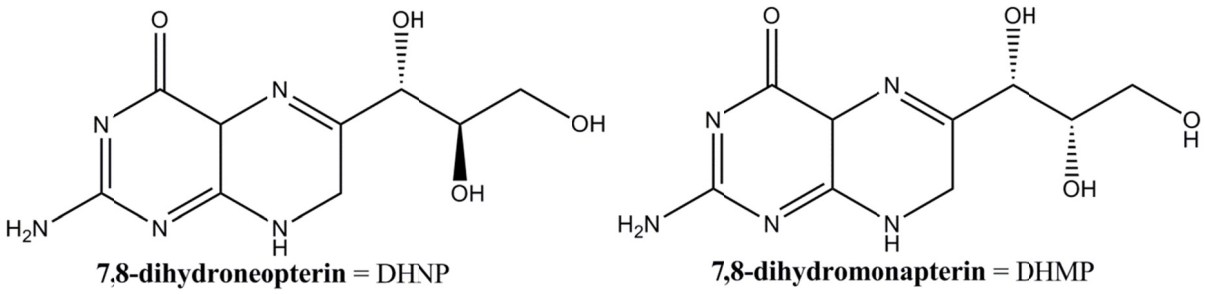


Retention time 4.5 min = DHNP

Retention time 5.5 min = DHMP

Retention time 6.8 min = HP

Retention time 8.3 min = DHXP



Positions of *Mt*DHNA residues are shown (Y54, K99, and E74)

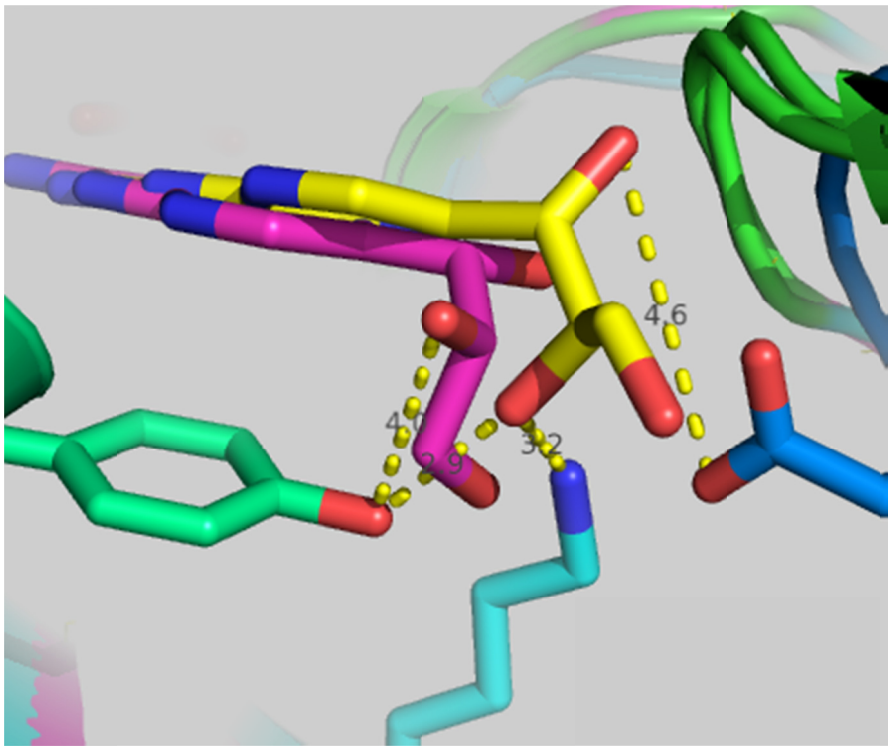
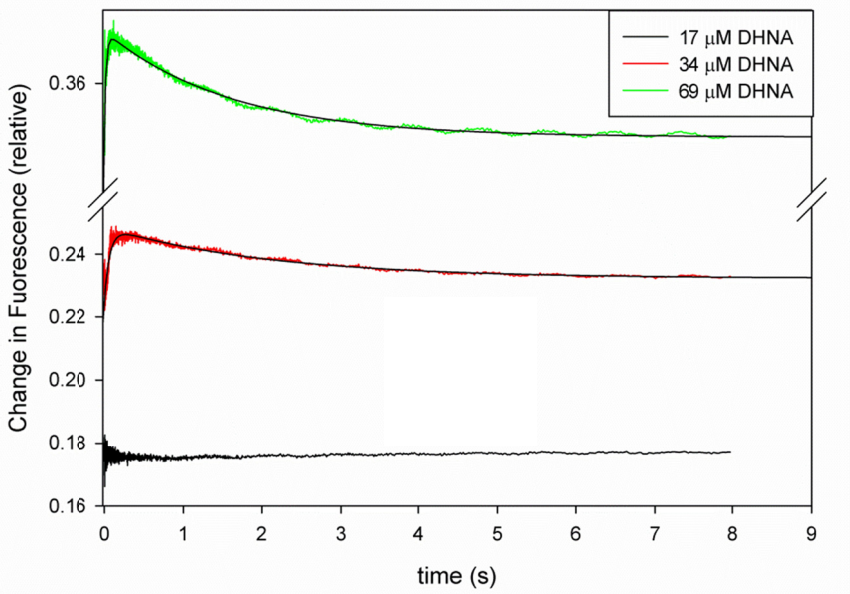


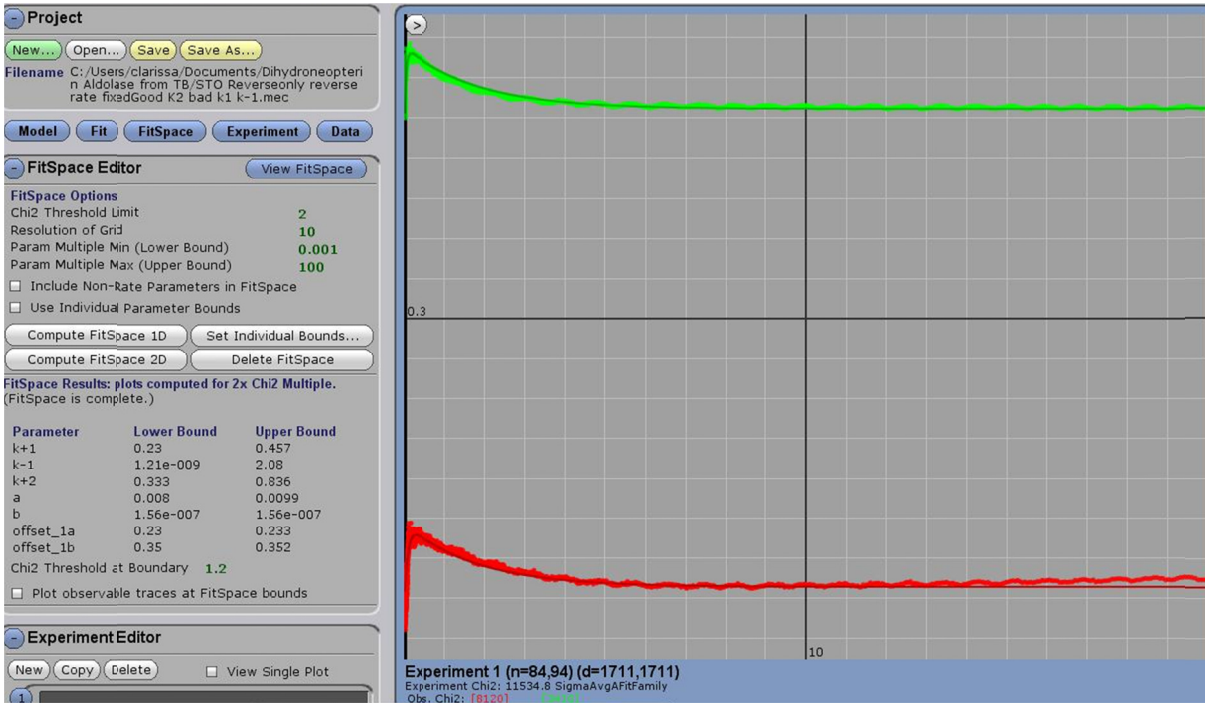
Figure S8: Comparison between SaDHNA active site with monapterin (magenta) or neopterin (yellow) bound[1]. K99 is shown in light blue, Y54 in green, and E74 in dark blue.

Figure S9: Reverse reaction single turnover

A)



B)



Single turnover experiments for the reverse reaction. 17, 34 or 69 μM *Mt*DHNA were mixed with 2 μM HP and 100 mM GA. Fluorescence signal was due to fluorescence of HP, so decrease in signal is expected as HP is consumed. A) Data were fitted to double exponential equations (k_{obs} = 0.3699 at 34 μM *Mt*DHNA, and k_{obs} = 0.6333 at 69 μM *Mt*DHNA). Data at 17 μM could not be fitted due to small amplitude of the second phase. B) Data fitting using Kintek Global Explorer, showing Fitspace results.

Figure S10: HPLC assays demonstrating that in the presence of *Mt*DHNA and HP, DHXP is the only product formed. Retention time 6.8 min = HP, and retention time 8.3 min = DHXP.

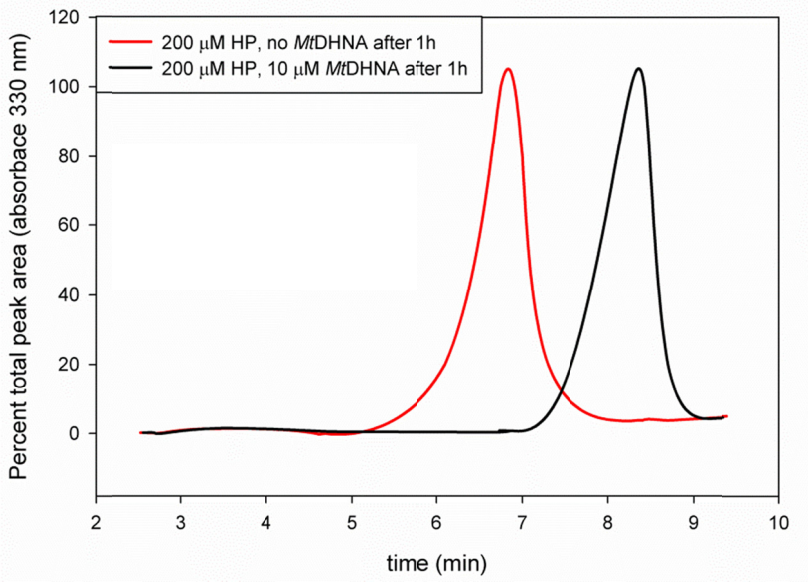


Table S1

Measured rates

Direction	Process	Rate or rate constant (s^{-1})	Method used to determine
Forward	DHNP consumption	$k_{burst\text{-}DHNP}$ = 0.0176 ± 0.0063 $k_{cat\text{-}DHNP}$ = 0.0112 ± 0.008	Quench-flow
		$k_{cat\text{-}DHNP}$ = 0.0146 ± 0.0015 s^{-1}	NMR
Forward	HP formation	$k_{burst\text{-}HP}$ = 0.037 ± 0.031 $k_{cat\text{-}HP}$ = 0.0091 ± 0.0008	Quench-flow
	-using DHNP	0.028 ± 0.001	Stopped-Flow (STO)
	-using DHMP	0.026 ± 0.001	Stopped-Flow (STO)
		$k_{cat\text{-}HP}$ = 0.0074 ± 0.0005 s^{-1}	NMR
Forward	DHMP formation	$k_{burst\text{-}DHMP}$ = 0.0098 ± 0.0023 $k_{cat\text{-}DHMP}$ = 0.00151 ± 0.0006	Quench-flow
		$k_{cat\text{-}DHMP}$ = 0.0015 ± 0.0004	NMR
Forward	DHXP formation	$k_{burst\text{-}DHXP}$ = 0.0652 ± 0.0267 $k_{cat\text{-}DHXP}$ = 0.00113 ± 0.0002	Quench-flow
		$k_{cat\text{-}DHXP}$ = 0.0023 ± 0.0004	NMR
Reverse	HP consumption	0.0247 ± 0.0012	NMR
		0.52 s^{-1} (estimated, see text)	Stopped-Flow (STO)
	DHNP formation	= 0.0128 ± 0.0004	NMR
	DHMP formation	= 0.0031 ± 0.0001	NMR

*STO = Single turnover conditions

Table S2: Models tested for dome-shaped proton inventory fit – Red letters indicate why the model was rejected

^{D2O}V						
Model	Equation	ϕ_1	Φ_2	$\Phi_{Solvent}$	R^2	$SKIE_{calc}$
1TS proton	$V_n = V_0*(1-n+n*\phi_1)$	0.76 ± 0.06			0.51	1.3
1 TS proton, 1 solvent proton	$V_n = V_0*(1-n+n*\phi_1)*(\Phi_{Solvent})^n$	0.18 ± 0.04		2.76 ± 0.33	0.87	2.01
2 TS protons $\phi_1 = \phi_2$	$V_n = V_0*(1-n+n*\phi_1)^2$	0.88 ± 0.03			0.50	1.3
2 TS protons, $\phi_1 = \phi_2$, solvent contribution	$V_n = V_0*(1-n+n*\phi_1)^2*(\Phi_{Solvent})^n$	2.61 ± 0.21		0.09 ± 0.02	0.90	1.63
2 TS protons $\phi_1 \neq \phi_2$	**$V_n = V_0*(1-n+n*\phi_1)*(1-n+n*\phi_2)$**	**1.88 ± 0.09**	**0.33 ± 0.03**		**0.84**	**1.6**
2 TS protons, $\phi_1 \neq \phi_2$, solvent contribution	$V_n = V_0*(1-n+n*\phi_1)*(1-n+n*\phi_2)*(\Phi_{Solvent})^n$	0.49 ± 0.27	2.55 ± 0.73	0.52 ± 0.40	0.71	1.53
2 distinct TS, one TS proton from each	$V_n = V_0*[(1-n+n*\phi_1) + (1-n+n*\phi_2)]$	0.20 ± 125415	0.0024 ± 125415		0	
1 TS proton, 1 RS proton	$V_n = V_0*[(1-n+n*\phi_1)/(1-n+n*\phi_2)]$	0.02 ± 0.06	0.08 ± 0.08		0.80	
2 TS protons, from each TS, weightening factor included (w_1)	$V_n = V_0*[w1*(1-n+n*\phi_{1a})*(1-n+n*\phi_{1b}) + (1-w1)*(1-n+n*\phi_{2a})*(1-n+n*\phi_{2b})]$	ϕ_{1a} = 1.04 ± 0.001 ϕ_{1b} = 0.98 ± 0.0002	Φ_{2a} = 0.62 ± 0.0002 Φ_{2b} = 0.97 ± 6704600		0.93 w_1 = 0.93	1.0

$^{D2O}V/K_{DHMP}$					
Model	Equation	ϕ_1	Φ_2	R^2	
1TS proton	$V_n = V_0*(1-n+n*\phi_1)$	0.33 ± 0.04		0.92	3.03
2 TS protons $\phi_1 \neq \phi_2$	**$V_n = V_0*(1-n+n*\phi_1)*(1-n+n*\phi_2)$**	**1.38 ± 0.23**	**0.18 ± 0.07**	**0.94**	**4.02**

References

(1) Blaszczyk, J.; Li, Y.; Gan, J.; Yan, H.; Ji, X. *J Mol Biol* **2007**, *368*, 161.