Supporting Information

Role of two conserved water molecules in the catalytic pocket of Thymidylate Synthase from *Mycobacterium tuberculosis*

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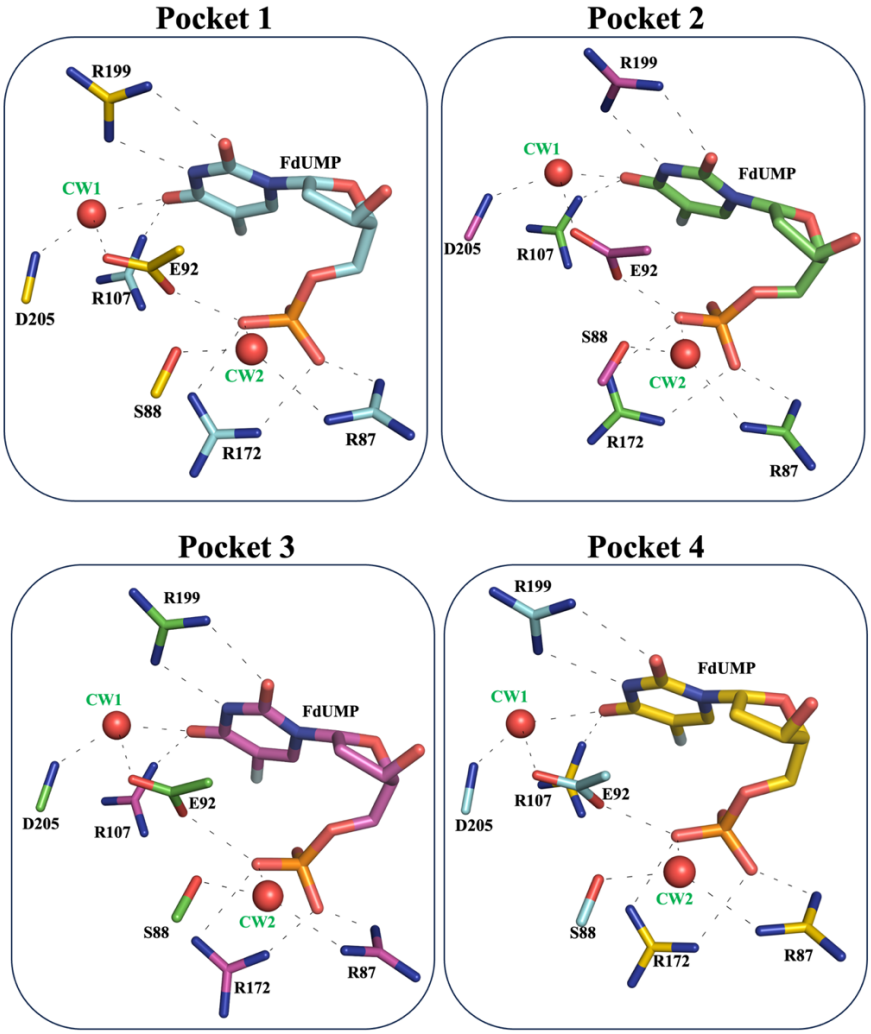
Table S1. Simulation setup and sampling.

System	Box dimensions ($Å^3$)	Number of $H_2O/Na^+/Cl^-$	Total no of atoms	Simulation	Sampling (ns) = Equilibration + Production	Trials
MtbThyX	115 × 11× 95	34054/129 /109	117915	Vanilla MD	10.5 + 500 = 510.5 ns	4
				Alchemical	5.5 + 260 = 265.5 ns	8
					5.5 + 310 = 315.5 ns	1
					5.5 + 205 = 210.5 ns	1
					5.5 + 520 = 525.5 ns	2
	117 × 117 ×117	49123/170 /150	164467	Alchemical	5.5 + 260 = 265.5 ns	2
Water Box	40.6 × 40.6 × 40.6	2334/7/7	7019	Alchemical	10.5 + 110 = 120.5 ns	3
					10.5 + 260 = 270.5 ns	5

Table S2. Key protein-ligand interaction distances in the MtbThyX pocket averaged (fourth column) from four trajectories (500 ns each) and compared with the X-ray data (third column).

Sl. No.	Bond	X-ray	MD
1.	R172:NH1 – UMP:O2P	2.93	2.98 ± 0.32
2.	E92:OE2 – UMP:O2P	2.51	3.10 ± 0.54
3.	R87:NH2 – UMP:O3P	2.80	3.02 ± 0.38
4.	R172:NH2 – UMP:O3P	3.10	2.89± 0.14
5.	R199:NH2 – UMP:O2	2.81	2.74 ± 0.00
6.	R199:NH1 – UMP:N3	2.85	2.86 ± 0.04
7.	R107:NH1 – UMP:O4	2.78	2.89 ± 0.19

Table S3. The zoomed-in view of the four pockets of MtbThyX indicated that the pockets are more or less identical. The timing of the initial exchange of water molecules with the bulk at the CW1 and CW2 locations in the four catalytic pockets of MtbThyX is presented in tabular form, based on various independent production simulations of 500 ns. The presence of water molecules without bulk water exchange throughout the trajectory is referred to as "present."



Location	Runs	Pocket 1	Pocket 2	Pocket 3	Pocket 4
CW1	Run 1	75 ns	172 ns	14 ns	Present
	Run 2	Present	320 ns	68 ns	100 ns
	Run 3	14 ns	Present	20 ns	55 ns
	Run 4	10 ns	Present	77 ns	170 ns
CW2	Run 1	119 ns	160 ns	167 ns	201 ns
	Run 2	Present	292 ns	5 ns	50 ns
	Run 3	170 ns	Present	72 ns	140 ns
	Run 4	42 ns	348ns	41 ns	260 ns

Table S4. Time taken for a water molecule to enter the empty catalytic pocket at the locations of CW1 and CW2 (read as dummy) of MtbThyX from various independent simulations.

Location	Runs	Pocket
CW1	Run 1	2 ns
	Run 2	1.5 ns
	Run 3	1.5 ns
	Run 4	500 ps
	Run 5	2 ns
CW2	Run 1	1 ns
	Run 2	680 ps
	Run 3	2 ns
	Run 4	200 ps
	Run 5	200 ps

Table S5. Free energy (in kcal/mol) change for the alchemical transformation in the water with restraint. Calculated using BAR. Error in s.e.m.

Alchemical Transformation	Replicas	ΔG^{free} (kcal/mol)	Average ΔG^{free} (kcal/mol)
WAT → DUM (11 windows)	Run 1	7.55 ± 0.01	7.55 ± 0.01
	Run 2	7.54 ± 0.01	
	Run 3	7.55 ± 0.01	
WAT → DUM (26 windows)	Run 1	7.55 ± 0.02	
	Run 2	7.55 ± 0.01	

Table S6. Free energy (in kcal/mol) change for the alchemical transformation in the water without any restraint. Calculated using BAR. Error in s.e.m.

Alchemical Transformation	Replicas	ΔG^{free} (kcal/mol)	Average ΔG^{free} (kcal/mol)
WAT → DUM (26 windows)	Run 1	6.22 ± 0.01	6.23 ± 0.01
	Run 2	6.24 ± 0.01	
	Run 3	6.24 ± 0.01	

Table S7. Free energy (in kcal/mol) change for the alchemical transformation in the protein with restraint around 3.4 Angstrom of the substrate(dUMP). Calculated using BAR. Error in s.e.m.

Alchemical Transformation (26 windows*10ns)	Replicas	ΔG^{comp} (kcal/mol)	Average ΔG^{comp} (kcal/mol)
CW1 → DUM	Run 1	20.04 ± 0.02	20.45 ± 0.24
	Run 2	20.15 ± 0.01	
	Run 3	20.49 ± 0.02	
	Run 4	21.14 ± 0.04	
CW2 → DUM	Run 1	13.00 ± 0.04	13.5 ± 0.25
	Run 2	13.80 ± 0.13	
	Run 3	13.7 ± 0.03	

Table S8. Free energy (in kcal/mol) change for the alchemical transformation in the protein without any restraint. Calculated using BAR. Error in s.e.m.

Alchemical Transformation	Replicas	ΔG^{comp} (kcal/mol)	Average ΔG^{comp} (kcal/mol)
CW1 → DUM (26 windows* 10ns/20ns)	Run 1	8.08 ± 0.1	8.45 ± 0.18
	Run 2	8.58 ± 0.1	
	Run 3	8.70 ± 0.1	
CW2 → DUM (11 windows*30ns)	Run 1	8.56 ± 0.2	8.46 ± 0.12
CW2 → DUM (26 windows*10ns)	Run 1	8.29 ± 0.04	
	Run 2	8.79 ± 0.15	
CW2 → DUM (41 windows*5ns)	Run 1	8.27 ± 0.26	

Table S9. Relative binding free affinity (ΔΔG in kcal/mol). ΔG^{comp} and ΔG^{free} are averaged from multiple independent trajectories (see Table S2 and Table S3). The error (s.e.m)associated with the ΔG^{comp} and ΔG^{free} were propagated and reported as error of ΔΔG.

	ΔG^{comp}	$\Delta\Delta G = \Delta G^{comp} - \Delta G^{free}$
Restraint within 3.4 Angstrom of dUMP		
CW1	20.47 ± 0.18	12.92 ± 0.18
CW2	13.5 ± 0.25	5.97 ± 0.25
No restraint		
CW1	8.45 ± 0.18	0.90 ± 0.18
CW2	8.46 ± 0.12	0.91 ± 0.12

Table S10. B-factors of CW1 and CW2 present in multiple crystallographic structures.

Sl. No.	PDB ID	Components	Organism	B-factors	
				CW1	CW2
1.	2AF6	*ThyX-FAD-BrdUMP*	*Mycobacterium tuberculosis*	22.14	18.7
2.	3GWC	*ThyX-FAD-FdUMP*	*Mycobacterium tuberculosis*	20.48	20.03
3.	1O25	*ThyX-dUMP*	*Thermogota maritima*	13.8	28.06
4.	1O26	*ThyX-FAD-dUMP*	*Thermogota maritima*	19.71	17.45
5.	1O28	*ThyX-FdUMP*	*Thermogota maritima*	19.58	20.31
6.	1O29	*ThyX-FAD-FdUMP*	*Thermogota maritima*	18.83	23.62
7.	1O24	*ThyX(apo)*	*Thermogota maritima*	28.68	38.29
8.	3AH5	*ThyX-FAD-dUMP*	*Helicobacter pylori*	15.47	18.80
9.	4P5A	ThyX-FAD-BrUMP	*Streptomyces cacaoi*	12.99	12.10
10.	4P5B	ThyX-FAD-BrdUMP	*Streptomyces cacaoi*	16.27	13.70

Table S11. Diffusion coefficients of CW1 and CW2 were calculated within a volume of 3.4 Å radius around the oxygen of uracil and the phosphate of dUMP, respectively. The average data from all four pockets were reported in the main text. The standard error of the mean (SEM) is presented as error.

Location	Pocket	D ($Å^2$/ps)	Average D
CW1	Pocket 1	0.0031 ± 0.0001	0.003 ± 0.0006
	Pocket 2	0.0050 ± 0.0012	
	Pocket 3	0.0026 ± 0.0002	
	Pocket 4	0.0023 ± 0.0005	
CW2	Pocket 1	0.0032 ± 0.0001	0.003 ± 0.0004
	Pocket 2	0.0030 ± 0.0001	
	Pocket 3	0.0036 ± 0.0001	
	Pocket 4	0.0017 ± 0.0001	
Bulk			0.63 ± 0.0002

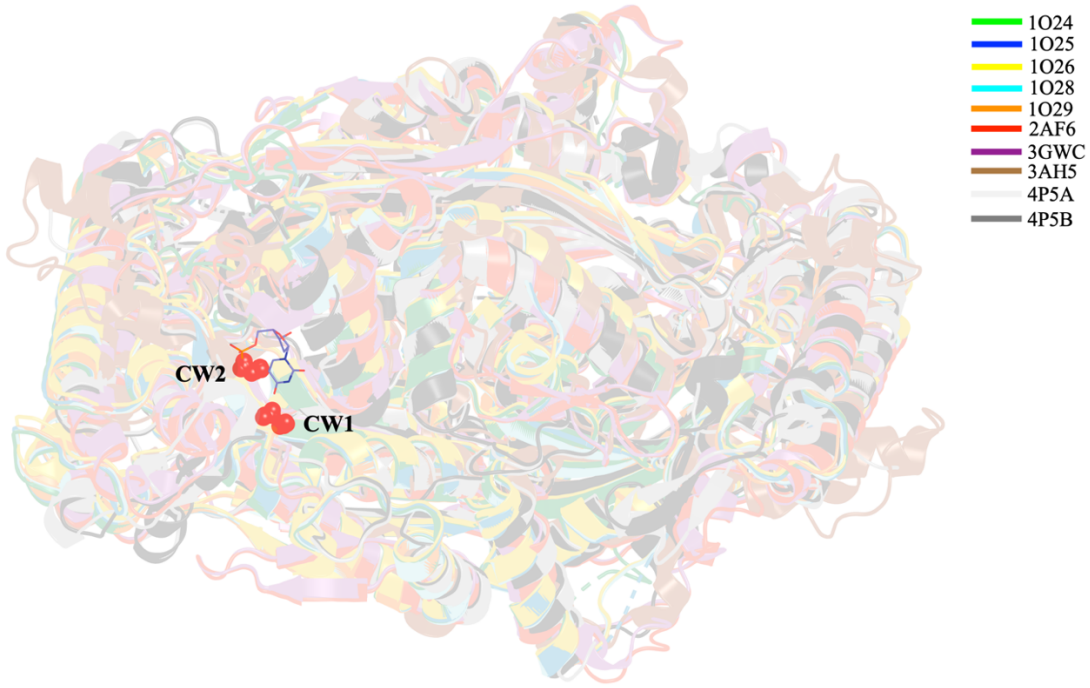


Figure S1. Alignment of multiple ThyX structures (different colors represent different PDB IDs) from *Mycobacterium tuberculosis*, *Thermotoga maritima*, *Helicobacter pylori* and *Streptomyces cacaoi*. The conserved water molecules (CW1 and CW2) are shown in red sphere.

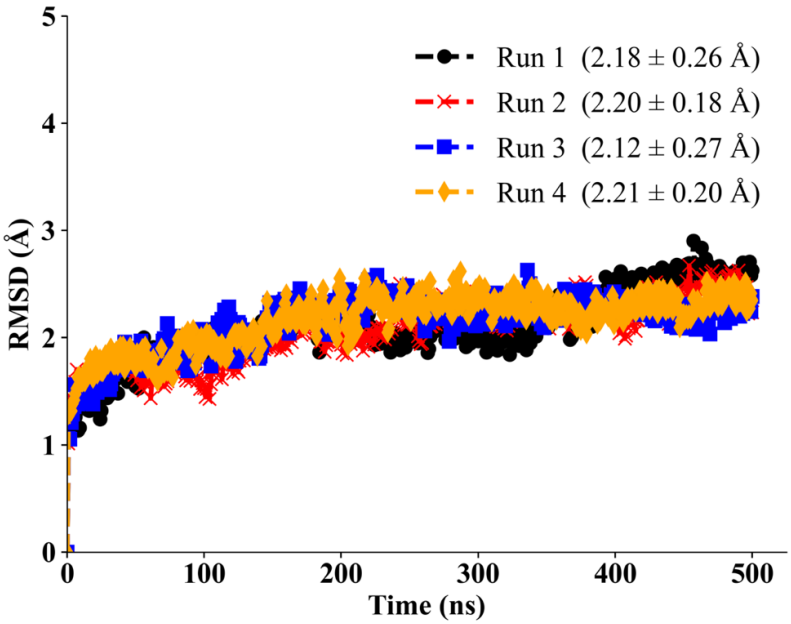


Figure S2. Protein backbone heavy atom RMSD versus time plot from four independent trajectories (MtbThyX complex). The average RMSD from the 450 ns trajectory is explicitly mentioned with the standard deviation as error.

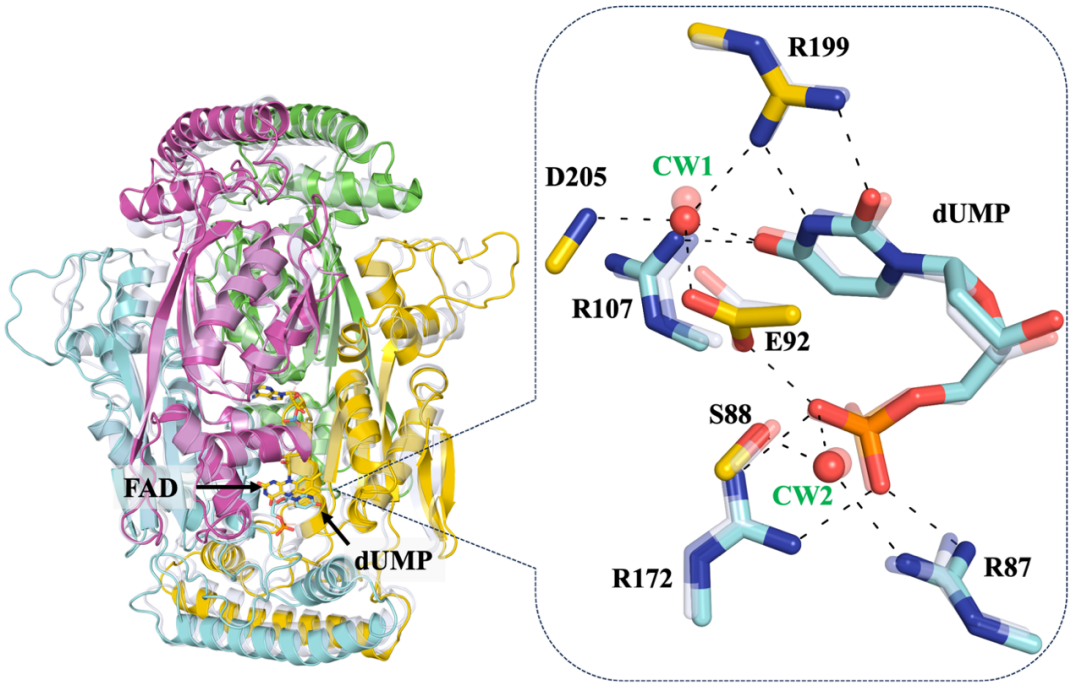


Figure S3. Overlay of the X-ray (in grey and transparent) and MD structure shows that protein-ligand interactions and conserved water molecules (CW1 and CW2) are largely identical in both the MD and X-ray structures (see zoomed-in view in the broken box).

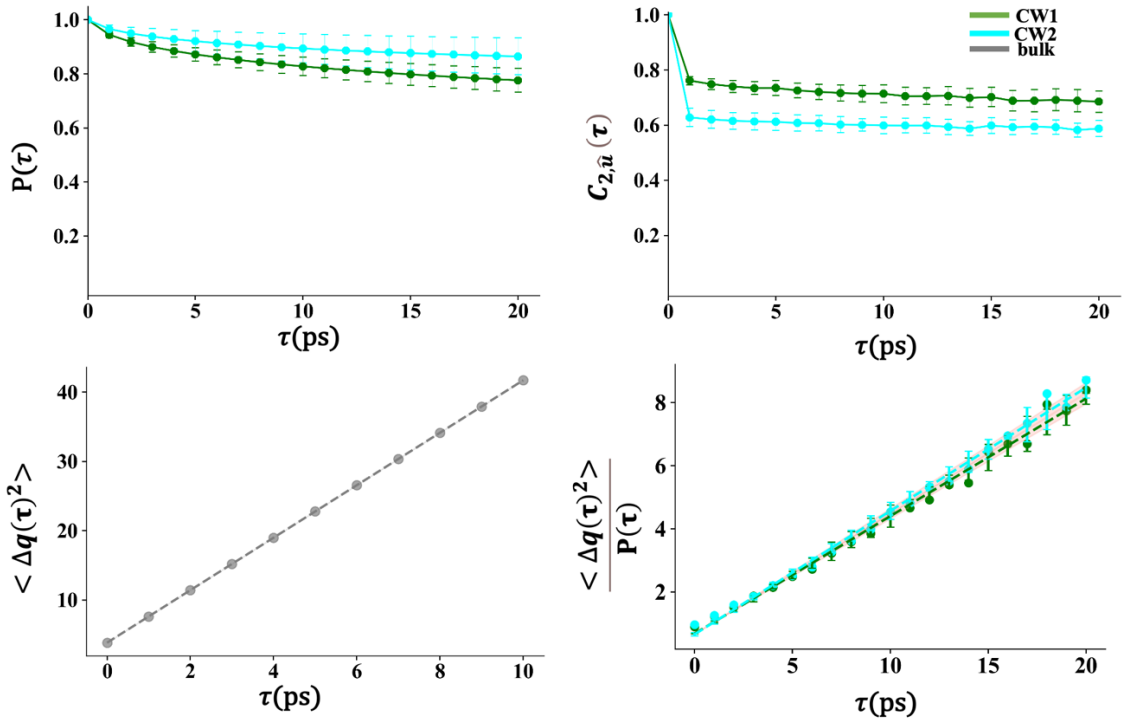


Figure S4. Extended data of Figure 2 of the main manuscript. Survival probabilities P(τ), orientational correlation time the water dipole $C_{2,\hat{u}}$ (τ) , mean-square-displacement <Δq(τ)^2> as a function of time. The colour coding represents different water molecules analysed in this study.

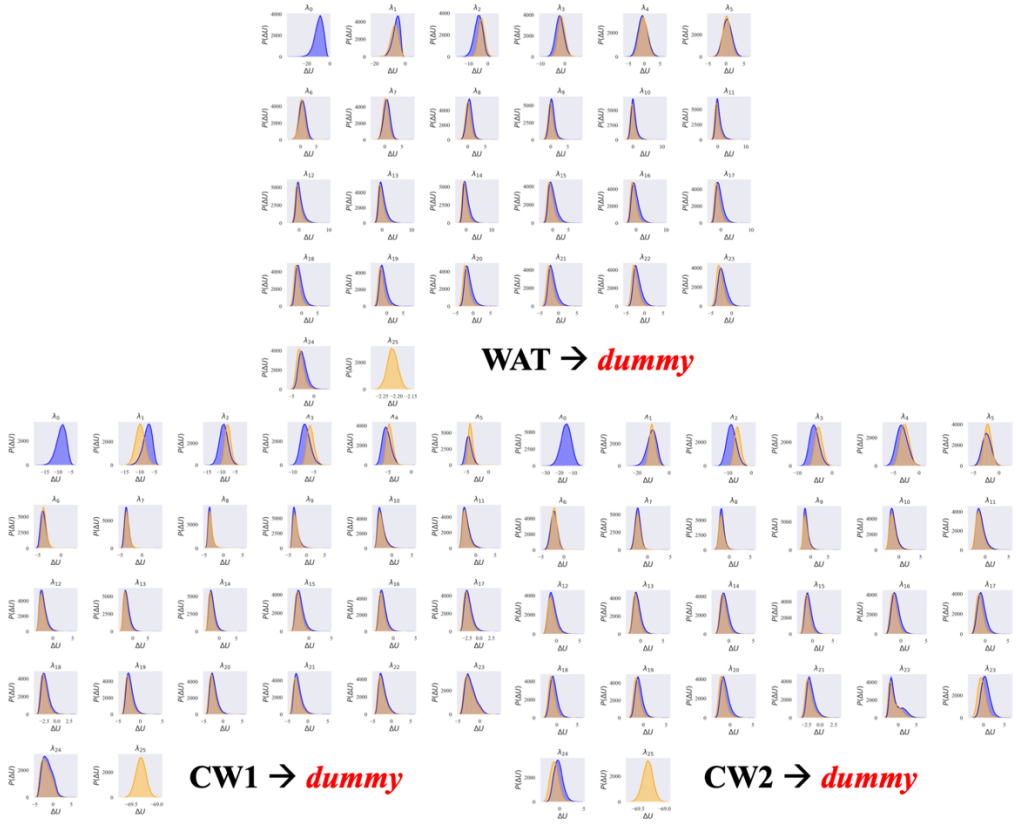


Figure S5. Alchemical simulation (26 λ windows, λ_0 and λ_{26} corresponds to the end states) free in water box. Probability distribution function is given in the Y-axis. The difference in potential energy (in kT, between two neighboring windows) is given in the X-axis. Probability distribution function for the forward (blue) and the backward (orange) transformations are highlighted. Note that, the significant overlap between the forward (forward) and the backward (orange) probability distributions ensured microscopic reversibility. Data given from a single alchemical trajectory.

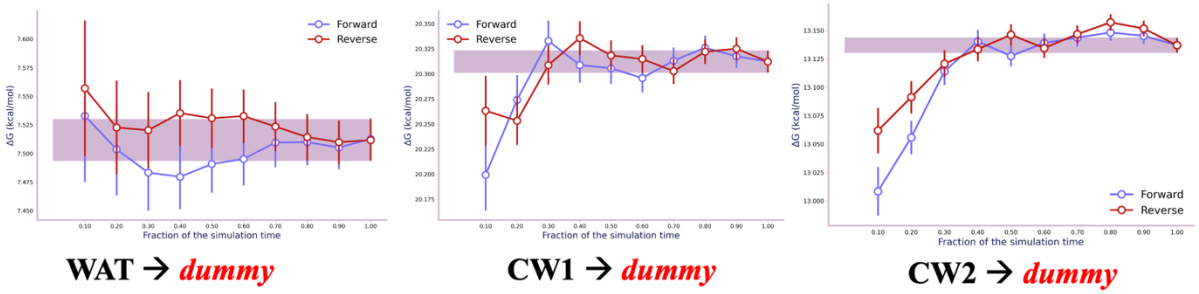


Figure S6. Time evolution of the free energies (with error bars) for WAT → DUM, CW1→DUM, CW2→DUM from left to right respectively. Data from all but 1ns of one of the trajectory were analyzed both chronologically ("forward") and in a time-reversed manner ("reverse").

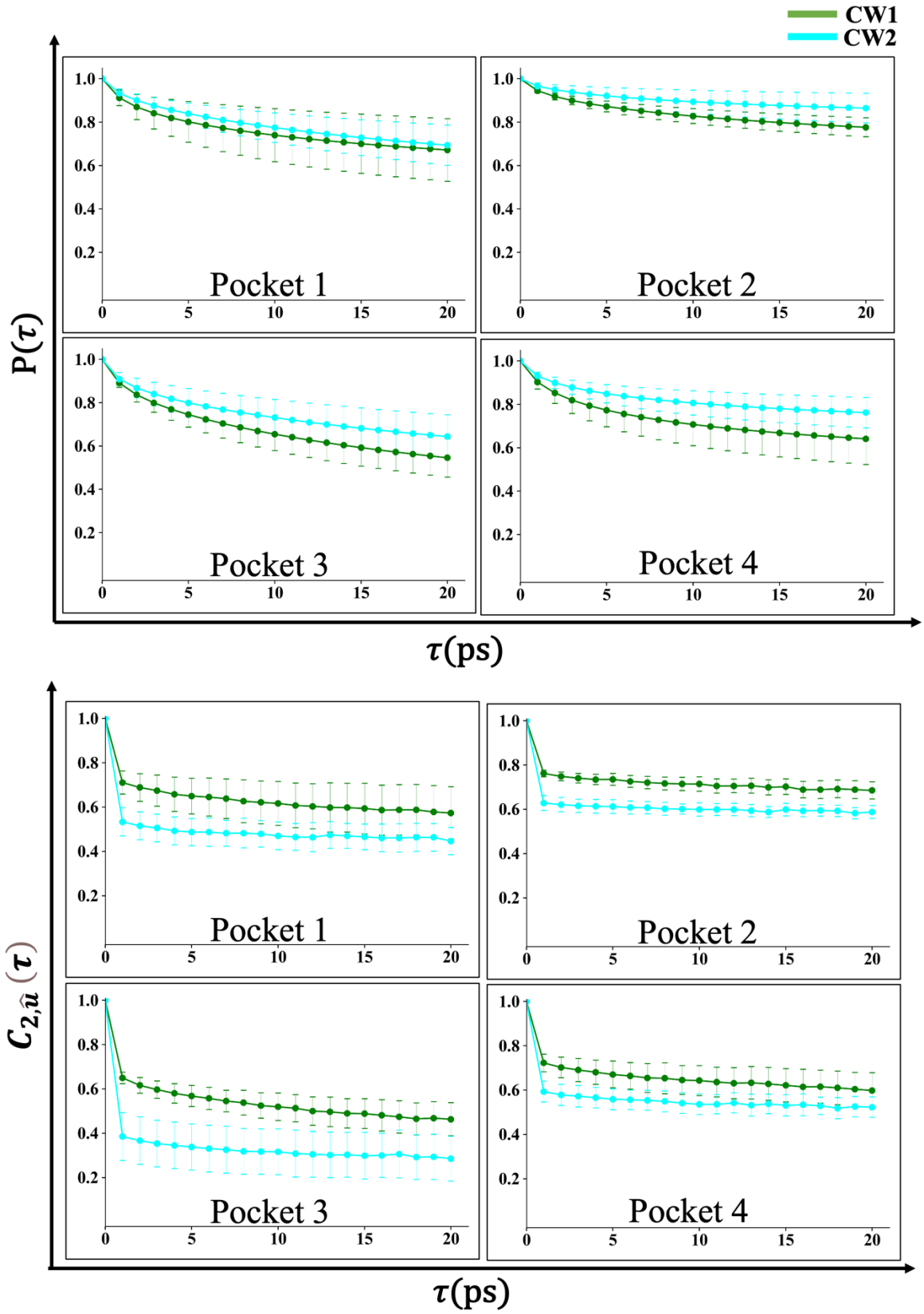


Figure S7. Survival probabilities P(τ)**,** orientational correlation time of the water dipole $C_{2,\hat{u}}$ (τ) for all four pockets as a function of time. The colour coding represents different water molecules analysed in this study.

Note 1 : Protein restraint and absolute binding affinity calculation

Water-dummy alchemical transformation and absolute binding affinity

In this study, we aimed to quantify the absolute binding affinity of conserved water molecules (CW1 and CW2) in the catalytic pocket of MtbThyX using alchemical simulations. The computations involved transforming the water molecules into dummy molecules, either within the protein (CW1 or CW2 → dummy) or in bulk water. Free energy, being a state function, the overall free energy change of the thermodynamic cycle is zero (Figure S8). Thus, the free energy difference between the upper (1a+1b) and lower horizontal (2a+2b) arm of Figure S8, depicted as $\Delta\Delta G$, should correspond to the left vertical arm (CW1 binding affinity to the protein pocket, i.e, ΔG_{bind}). It is noteworthy that the right-hand vertical arm gives $\Delta G_{bind} = 0$ as the dummy does not have any interaction with the surroundings. However, it is absolutely crucial for the transformation to occur within the protein pocket since the reverse process (dummy → CW1 or CW2) needs to regenerate the water molecule at the conserved site and satisfy the criteria for microscopic reversibility. To achieve this, we must apply restraints to the water molecules. Without these restraints, the alchemical molecules may drift away from the positions of the conserved water molecules (labelled CW1 and CW2), which would make the simulation less reflective of the actual process. Implementing restraints enhances the sampling efficiency by preventing exploration of irrelevant regions of phase space and improves the convergence of the calculated free energy.[1] Applying restraints during the transformation from water to dummy molecules is essential for relevant sampling and for estimating accurate free energy values.[2] Another crucial aspect of accurate free energy calculation is stratification.[3] Increased stratification, or multistage sampling, minimises systematic errors in the calculated free energy. We have already established the convergence of the calculated free energy from various sampling strategies (Table S8) and multiple independent MD runs (Table S5-S7).

In this work, first, we used positional harmonic restraint on the water molecule (say CW1), which was then slowly transformed into a dummy molecule (Figure S8). However, the restraint is only on the water molecule; the protein allows a single bulk water entry by opening the channel lid. We also observed translation of the protein; as a result, the dummy molecule was eventually found away from the catalytic pocket of MtbThyX, as schematically shown in the right section of the upper horizontal arm of Figure S8. In summary, at the end of water-dummy transformation, a water molecule from the bulk occupied the position of CW1 (thus, different from the desired state where the dummy is created at the CW1 location of the protein). Due to this reason, the $\Delta\Delta G \sim 0$, as both the vertical arms of Figure S8 correspond to water binding to the catalytic pocket, thus $\Delta\Delta G \neq \Delta G_{bind}$.

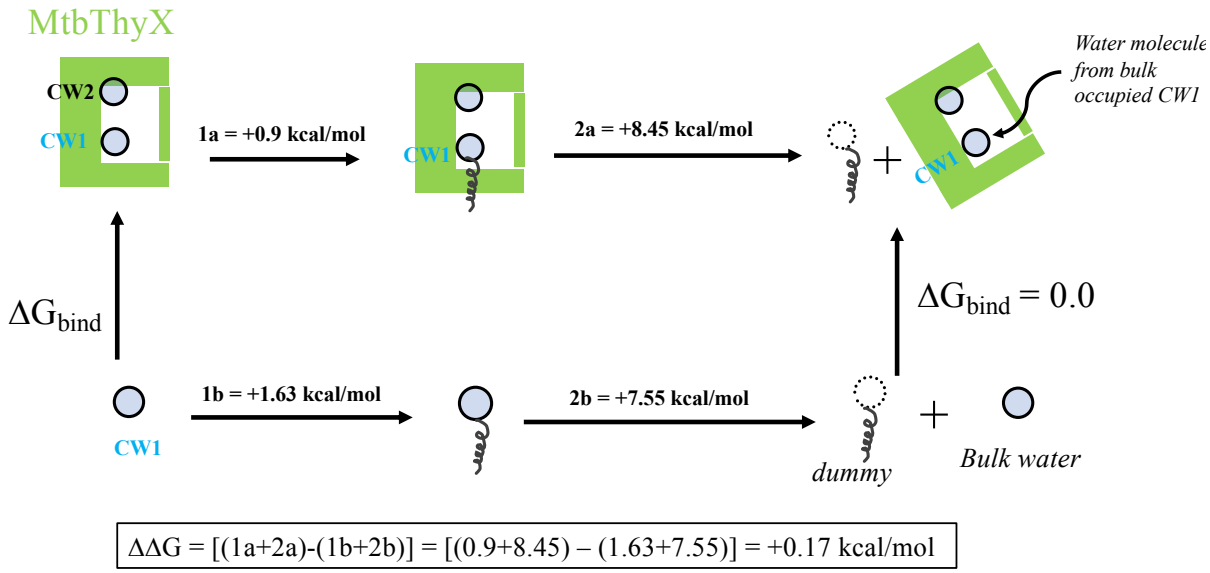


Figure S8. Water-dummy alchemical transformation without restraining the protein. The free energy cost for legs 1a and 1b was estimated using equations 3 and 4 from the main text. The free energy changes for legs 2a and 2b were computed through alchemical simulations. The data is averaged over three MD replicas.

This result clearly indicates that for a zero free energy of zero along the right vertical arm (dummy binding to the CW1 location of the protein), we need to apply restraints in the catalytic pocket. This will prevent the channel lid from opening and stop any water molecules from entering the channel. Additionally, applying restraint to both the water and the catalytic pocket will ensure that the protein does not change its conformation by the end of the alchemical simulations, and the dummy is created at the CW1 location. Thus, we applied harmonic restraint to the catalytic pocket of MtbThyX (see main text) and removed the retraining free energy contributions either analytically or numerically by post-processing the trajectories (see main text).

References

1. Mey, Antonia SJS, Bryce K. Allen, Hannah E. Bruce Macdonald, John D. Chodera, David F. Hahn, Maximilian Kuhn, Julien Michel et al. "Best practices for alchemical free energy calculations [article v1. 0]." *Living journal of computational molecular science* 2, no. 1 (2020): 18378.

2. Clark, Finlay, Graeme Robb, Daniel J. Cole, and Julien Michel. "Comparison of receptor–ligand restraint schemes for alchemical absolute binding free energy calculations." *Journal of Chemical Theory and Computation* 19, no. 12 (2023): 3686-3704.

3. Pohorille, Andrew, Christopher Jarzynski, and Christophe Chipot. "Good practices in free-energy calculations." *The Journal of Physical Chemistry B* 114, no. 32 (2010): 10235-10253.

Force field parameters of dUMP$^{(d)}$ generated by CGenFF

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    9   11   12    5 ;   CG3C51    CG3C52    CG3C51
    9   11   26    5 ;   CG3C51    CG3C52     HGA2
    9   11   27    5 ;   CG3C51    CG3C52     HGA2
   12   11   26    5 ;   CG3C51    CG3C52     HGA2
   12   11   27    5 ;   CG3C51    CG3C52     HGA2
   26   11   27    5 ;    HGA2     CG3C52     HGA2
    8   12   11    5 ;   OG3C51    CG3C51    CG3C52
    8   12   13    5 ;   OG3C51    CG3C51    NG2R61
    8   12   28    5 ;   OG3C51    CG3C51     HGA1
   11   12   13    5 ;   CG3C52    CG3C51    NG2R61
   11   12   28    5 ;   CG3C52    CG3C51     HGA1
   13   12   28    5 ;   NG2R61    CG3C51     HGA1
   12   13   14    5 ;   CG3C51    NG2R61    CG2R63
   12   13   20    5 ;   CG3C51    NG2R61    CG2R62
   14   13   20    5 ;   CG2R63    NG2R61    CG2R62
   13   14   15    5 ;   NG2R61    CG2R63     OG2D4
   13   14   16    5 ;   NG2R61    CG2R63    NG2R62
   15   14   16    5 ;    OG2D4     CG2R63    NG2R62
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 14   16   17 5 ;   CG2R63   NG2R62   CG2R62
 16   17   18 5 ;   NG2R62   CG2R62   OG312
 16   17   19 5 ;   NG2R62   CG2R62   CG2R62
 18   17   19 5 ;   OG312   CG2R62   CG2R62
 17   19   20 5 ;   CG2R62   CG2R62   CG2R62
 17   19   30 5 ;   CG2R62   CG2R62   HGR62
 20   19   30 5 ;   CG2R62   CG2R62   HGR62
 13   20   19 5 ;   NG2R61   CG2R62   CG2R62
 13   20   29 5 ;   NG2R61   CG2R62   HGR62
 19   20   29 5 ;   CG2R62   CG2R62   HGR62

[ dihedrals ]
;        ai        aj      ak        al funct                          c0                        c1
                   c2                          c3                      c4                        c5
  2    1    6    5       9 ;   OG2P1    PG2    OG303    CG321
  3    1    6    5       9 ;   OG2P1    PG2    OG303    CG321
  4    1    6    5       9 ;   OG2P1    PG2    OG303    CG321
  7    5    6    1       9 ;   CG3C51    CG321    OG303    PG2
 21    5    6    1       9 ;   HGA2    CG321    OG303    PG2
 22    5    6    1       9 ;   HGA2    CG321    OG303    PG2
  6    5    7    8       9 ;   OG303    CG321    CG3C51    OG3C51
  6    5    7    9       9 ;   OG303    CG321    CG3C51    CG3C51
  6    5    7   23       9 ;   OG303    CG321    CG3C51    HGA1
 21    5    7    8       9 ;   HGA2    CG321    CG3C51    OG3C51
 21    5    7    9       9 ;   HGA2    CG321    CG3C51    CG3C51
 21    5    7   23       9 ;   HGA2    CG321    CG3C51    HGA1
 22    5    7    8       9 ;   HGA2    CG321    CG3C51    OG3C51
 22    5    7    9       9 ;   HGA2    CG321    CG3C51    CG3C51
 22    5    7   23       9 ;   HGA2    CG321    CG3C51    HGA1
  5    7    8   12       9 ;   CG321    CG3C51    OG3C51    CG3C51
  9    7    8   12       9 ;   CG3C51    CG3C51    OG3C51    CG3C51
 23    7    8   12       9 ;   HGA1    CG3C51    OG3C51    CG3C51
  5    7    9   10       9 ;   CG321    CG3C51    CG3C51    OG311
  5    7    9   11       9 ;   CG321    CG3C51    CG3C51    CG3C52
  5    7    9   24       9 ;   CG321    CG3C51    CG3C51    HGA1
  8    7    9   10       9 ;   OG3C51    CG3C51    CG3C51    OG311
  8    7    9   11       9 ;   OG3C51    CG3C51    CG3C51    CG3C52
  8    7    9   24       9 ;   OG3C51    CG3C51    CG3C51    HGA1
 23    7    9   10       9 ;   HGA1    CG3C51    CG3C51    OG311
 23    7    9   11       9 ;   HGA1    CG3C51    CG3C51    CG3C52
 23    7    9   24       9 ;   HGA1    CG3C51    CG3C51    HGA1
  7    8   12   11       9 ;   CG3C51    OG3C51    CG3C51    CG3C52
  7    8   12   13       9 ;   CG3C51    OG3C51    CG3C51    NG2R61
  7    8   12   28       9 ;   CG3C51    OG3C51    CG3C51    HGA1
  7    9   10   25       9 ;   CG3C51    CG3C51    OG311    HGP1
 11    9   10   25       9 ;   CG3C52    CG3C51    OG311    HGP1
 24    9   10   25       9 ;   HGA1    CG3C51    OG311    HGP1
  7    9   11   12       9 ;   CG3C51    CG3C51    CG3C52    CG3C51
  7    9   11   26       9 ;   CG3C51    CG3C51    CG3C52    HGA2
  7    9   11   27       9 ;   CG3C51    CG3C51    CG3C52    HGA2
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 10    9   11   12     9 ;    OG311    CG3C51    CG3C52    CG3C51
 10    9   11   26     9 ;    OG311    CG3C51    CG3C52     HGA2
 10    9   11   27     9 ;    OG311    CG3C51    CG3C52     HGA2
 24    9   11   12     9 ;     HGA1    CG3C51    CG3C52    CG3C51
 24    9   11   26     9 ;     HGA1    CG3C51    CG3C52     HGA2
 24    9   11   27     9 ;     HGA1    CG3C51    CG3C52     HGA2
  9   11   12    8     9 ;   CG3C51    CG3C52    CG3C51    OG3C51
  9   11   12   13     9 ;   CG3C51    CG3C52    CG3C51    NG2R61
  9   11   12   28     9 ;   CG3C51    CG3C52    CG3C51     HGA1
 26   11   12    8     9 ;    HGA2    CG3C52    CG3C51    OG3C51
 26   11   12   13     9 ;    HGA2    CG3C52    CG3C51    NG2R61
 26   11   12   28     9 ;    HGA2    CG3C52    CG3C51     HGA1
 27   11   12    8     9 ;    HGA2    CG3C52    CG3C51    OG3C51
 27   11   12   13     9 ;    HGA2    CG3C52    CG3C51    NG2R61
 27   11   12   28     9 ;    HGA2    CG3C52    CG3C51     HGA1
  8   12   13   14     9 ;   OG3C51    CG3C51    NG2R61    CG2R63
  8   12   13   20     9 ;   OG3C51    CG3C51    NG2R61    CG2R62
 11   12   13   14     9 ;   CG3C52    CG3C51    NG2R61    CG2R63
 11   12   13   20     9 ;   CG3C52    CG3C51    NG2R61    CG2R62
 28   12   13   14     9 ;    HGA1    CG3C51    NG2R61    CG2R63
 28   12   13   20     9 ;    HGA1    CG3C51    NG2R61    CG2R62
 12   13   14   15     9 ;   CG3C51    NG2R61    CG2R63     OG2D4
 12   13   14   16     9 ;   CG3C51    NG2R61    CG2R63    NG2R62
 20   13   14   15     9 ;   CG2R62    NG2R61    CG2R63     OG2D4
 20   13   14   16     9 ;   CG2R62    NG2R61    CG2R63    NG2R62
 12   13   20   19     9 ;   CG3C51    NG2R61    CG2R62    CG2R62
 12   13   20   29     9 ;   CG3C51    NG2R61    CG2R62     HGR62
 14   13   20   19     9 ;   CG2R63    NG2R61    CG2R62    CG2R62
 14   13   20   29     9 ;   CG2R63    NG2R61    CG2R62     HGR62
 13   14   16   17     9 ;   NG2R61    CG2R63    NG2R62    CG2R62
 15   14   16   17     9 ;    OG2D4    CG2R63    NG2R62    CG2R62
 14   16   17   18     9 ;   CG2R63    NG2R62    CG2R62     OG312
 14   16   17   19     9 ;   CG2R63    NG2R62    CG2R62    CG2R62
 16   17   19   20     9 ;   NG2R62    CG2R62    CG2R62    CG2R62
 16   17   19   30     9 ;   NG2R62    CG2R62    CG2R62     HGR62
 18   17   19   20     9 ;    OG312    CG2R62    CG2R62    CG2R62
 18   17   19   30     9 ;    OG312    CG2R62    CG2R62     HGR62
 17   19   20   13     9 ;   CG2R62    CG2R62    CG2R62    NG2R61
 17   19   20   29     9 ;   CG2R62    CG2R62    CG2R62     HGR62
 30   19   20   13     9 ;    HGR62    CG2R62    CG2R62    NG2R61
 30   19   20   29     9 ;    HGR62    CG2R62    CG2R62     HGR62

[ dihedrals ]
;        ai        aj      ak        al funct                c0                c1
                  c2                c3
  14    13   16   15     2
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[ bondtypes ]
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;     i       j  func          b0          kb
  CG2R62    OG312     1   0.12600000    439320.00


[ angletypes ]
;     i       j       k  func       theta0       ktheta          ub0          kub
  CG2R62   CG2R62    OG312     5   120.000000   334.720000   0.00000000         0.00
  NG2R62   CG2R62    OG312     5   121.000000   744.752000   0.23080000     75312.00


[ dihedraltypes ]
;     i       j       k       l  func         phi0         kphi  mult
  CG2R62   CG2R62   CG2R62    OG312     9   180.000000    12.970400     2
   OG312   CG2R62   CG2R62    HGR62     9   180.000000    10.041600     2
   OG312   CG2R62   NG2R62   CG2R63     9   180.000000    12.552000     2


[ dihedraltypes ]
; 'improper' dihedrals
;     i       j       k       l  func         phi0         kphi
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