Supporting Information

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Materials and Methods

Preparation and Crystallization of the Hydrogenase Pleiotropically Acting Protein in the Nucleotide-Bound States. The overexpression and purification of hydrogenase pleiotropically acting protein (Hyp)E from *Thermococcus kodakarensis* (TkHypE) were performed as described previously (1). *Escherichia coli* cells producing TkHypE were sonicated on ice, and the supernatant after centrifugation (34,600 × *g*, 30 min at 277 K) was subjected to heat treatment at 358 K for 15 min. After centrifugation (34,600 × *g*, 30 min at 277 K), the supernatant was applied to an anion-exchange column (HiTrap Q HP; GE Healthcare) equilibrated with 50 mM Tris–HCl pH 8.0 and 1 mM DTT. Proteins were eluted with a linear gradient of NaCl from 0.15 to 0.5 M. The sample was then applied to a gel-filtration column (Superdex200 10/300 GL, GE Healthcare) equilibrated with 20 mM Tris–HCl pH 7.5, 150 mM NaCl, and 1 mM tris (2-carboxyethyl) phosphine (TCEP).

The protein sample for crystallization was concentrated to 20 mg mL^{-1} in 20 mM Mes–NaOH pH 6.0, 5 mM $MgCl_2$, and 1 mM TCEP. The sample was mixed with 10 mM KOCN and incubated for 4 h at room temperature or for 20 h at 277 K. Then, 5 mM adenosine 5′-(β,γ-imido)triphosphate (AMPPNP) or 10 mM ATP was added. The sample with AMPPNP was incubated for 2 h at room temperature. The sample with ATP was immediately used for crystallization.

Crystallization was performed by the sitting-drop vapor-diffusion method at 293 K using 96-well Intelli-plates or CrystalClear D Strips (Hampton Research). Cluster crystals of HypE were initially obtained using Crystal Screen Cryo (Hampton Research) condition 39 [0.085 M Hepes–NaOH pH 7.5, 1.7 M ammonium sulfate, 1.7% (vol/vol) polyethylene glycol 400, 15% (vol/vol) glycerol]. The crystallization condition was optimized by varying the pH and the concentration of ammonium sulfate and glycerol. Addition of the Additive Screen reagent 69 [20% (wt/vol) benzamidine hydrochloride] (Hampton Research) inhibited the cluster formation. Finally, crystals suitable for data collection were obtained using reservoir solution containing 0.1 M Mes–NaOH pH 6.0, 1.4–1.55 M ammonium sulfate, 1.7% (vol/vol) polyethylene glycol 400, and 6% (vol/vol) glycerol. Drops were prepared by mixing 1.0 μL protein, 0.8 μL reservoir solution, and 0.2 μL of the additive screen reagent.

Data Collection and Structure Determination. Crystals were soaked in reservoir solution including 18% (vol/vol) glycerol for cryoprotection and then flash-cooled in a nitrogen stream at 100 K. The dataset "ATP pH7.0" was collected from a crystal grown in drops made by mixing the protein sample in 20 mM Hepes pH 7.0, 5 mM $MgCl_2$, 1 mM TCEP, 10 mM ATP, and the above described reservoir solution. Diffraction datasets were collected at the beamlines, BL-1A, BL-5A, and AR-NW12A (Photon Factory). All data were processed with *XDS* (2). The structures were determined by the molecular replacement method with *MOLREP* (3), using the previously determined HypE structure in its ternary complex (PDB entry 3VYT). The models were refined with the programs *CNS* (4), *Refmac5* (5), and *PHENIX* (6) and were validated with Molprobity (7).

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2. Kabsch W (2010) Xds. *Acta Crystallogr D Biol Crystallogr* 66(Pt 2):125–132.
3. Vagin A, Teplyakov A (2010) An approach to multi-copy search in molecular replacement. *Acta Crystallogr D Biol Crystallogr* 66(Pt 1):22–25.
4. Brünger AT, et al. (1998) Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr D Biol Crystallogr* 54(Pt 5): 905–921.
5. Murshudov GN, Vagin AA, Dodson EJ (1997) Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr* 53(Pt 3): 240–255.
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7. Chen VB, et al. (2010) MolProbity: All-atom structure validation for macromolecular crystallography. *Acta Crystallogr D Biol Crystallogr* 66(Pt 1):12–21.

Fig. S1. Sequence alignment and secondary structure assignment of HypE proteins from *Thermococcus kodakarensis*, *Pyrococcus furiosus*, *E. coli*, *Shigella boydii*, *Rhodobacter capsulatus*, *Azorhizobium caulinodans*, *Rhizobium leguminosarum*, *Synechococcus sp*. PCC 7002, *Bradyrhizobium japonicum*, *Thiocapsa roseopersicina*, *Ralstonia eutropha*, *Pyrococcus abyssi*, *Methanospirillum hungatei*, *Archaeoglobus fulgidus*, *Methanocaldococcus jannaschii*, *Desulfovibrio vulgaris*, and aminoimidazole ribonucleotide synthetase (PurM) family members: thiamin-monophosphate kinase (ThiL), selenophosphate synthase (SelD), and phosphoribosylaminoimidazole synthetase (PurM).

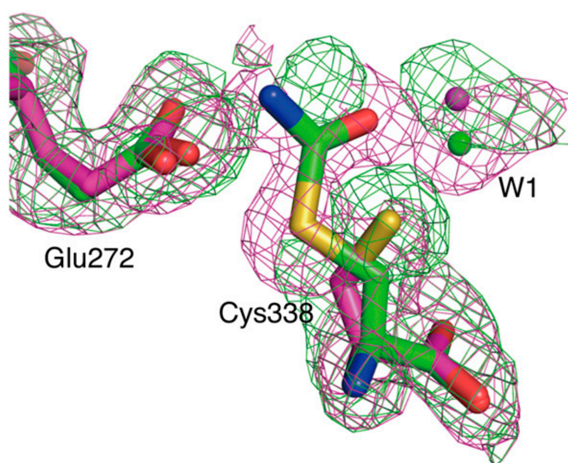

Fig. S2. Conformational changes surrounding Cys338 in the carbamoylated and unreacted states. Carbon atoms, W1, and F_o–F_c maps of carbamoylated and unreacted states are shown in green and magenta, respectively. The F_o–F_c maps are shown in mesh at 2.5 σ.

Table S1. Data collection and refinement statistics

	AMPPNP	ATP	ATP pH7.0
	Data collection		
Space group	$P4_32_12$	$P4_32_12$	$P4_32_12$
Cell dimensions			
a, *b*, *c* (Å)	102.9, 102.9, 104.0	102.9, 102.9, 104.3	102.4, 102.4. 105.5
Resolution (Å)*	50–1.53 (1.63–1.53)	50–1.64 (1.74–1.64)	47–1.86 (1.98–1.86)
R_{sym} (%)*,†	7.6 (60.9)	6.7 (72.2)	7.4 (66.6)
$I/\sigma(I)$	22.9 (5.2)	27.7 (4.5)	22.1 (4.5)
Completeness (%)*	99.6 (98.9)	99.8 (99.6)	99.6 (98.0)
	Refinement		
Resolution (Å)	42–1.53	42–1.64	47–1.86
No. reflections	83,607	68,178	47,503
R_{work}/R_{free} (%)‡,§	17.0/18.4	17.5/19.6	17.3/20.0
Rms deviations			
Bond length (Å)	0.06	0.08	0.008
Bond angles (°)	1.18	1.18	1.13
Ramachandran plot			
Preferred regions	97.5	98.3	97.6
Allowed regions	1.9	1.1	1.8
Outliers	0.6	0.6	0.6

*Values in parentheses are for the high-resolution shell.

†R_{sym} = S| I_h-<I_h>|/S<I_h>, where I_h is the observed intensity and <I_h> is the average intensity over symmetry-equivalent measurements.

‡R_{work}= S|F_o–F_c|/S|F_o|, where F_c is calculated structure factor.

§R_{free} is the same as R_{work}, but calculated for 5% of randomly chosen reflections that were omitted in the refinement.