SUPPLEMENTAL DATA

Structure and functional properties of *Bacillus subtilis* endospore-biogenesis factor StoA

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Purification of non-tagged sStoA– *E. coli* TOP10/pLMC19 was grown in LB medium at 37 °C. At $OD_{600} \approx 0.5$ *stoA* expression was induced by adding L-arabinose (0.02% (w/v), final concentration). After incubation for 3 h, cells were collected by centrifugation, washed and suspended in 20 mM Tris-HCl, pH 8.0, containing 1 mM DTT and broken by passage (three times) through a French pressure cell operated at 18,000 Psi. The lysate was centrifuged at 48,000 × g for 40 min at 4 °C and the supernatant was centrifuged at 100,000 × g for 1 h at 4 °C. The resulting supernatant was applied to a HiLoad 26/10 Q Sepharose HP anion exchange column (GE healthcare) equilibrated with 20 mM Tris-HCl, pH 8.0, containing 1 mM DTT. Proteins were eluted using a gradient of 0 – 1 M NaCl in the same buffer. Fractions containing sStoA were pooled, buffer exchanged into 20 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1 mM DTT using an Amicon Ultra 15 centrifugal filter (10 kDa M_W cut-off; Millipore) and applied to a Sephacryl S-100 HR gel filtration column (2.6 cm × 100 cm; GE Healthcare), equilibrated with 20 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1 mM DTT. Fractions containing sStoA were concentrated and buffer exchanged into 20 mM Tris-HCl, pH 8.0 as described above. The preparation was used in intial crystallisation trials and to generate a StoA antiserum.

sStoA reduction potential determination– The midpoint reduction potential of sStoA was determined by calculating the fraction of sStoA reduced, f_r, at each point in the fluorescence titration (data at 344 nm) and plotting this as a function of the system potential. The data were fitted to the Nernst equation (Equ. S1), in which E_m, the midpoint reduction potential of sStoA, and n, the number of electrons, were allowed to float. F is the Faraday constant, R is the gas constant, T is the temperature and E_h is the standard reduction potential of the DTT couple, which was taken to be -330 mV at pH 7 and 25 °C (1).

$$f_r = \frac{\exp^{(E_m - E_h)nF/RT}}{1 + \exp^{(E_m - E_h)nF/RT}} \qquad \text{(S1)}$$

*Determination of sStoA pK_a value*s– Following the reaction of cysteine side chains with alkylating reagents is a well established method for determining the pK_a values of protein cysteine thiol groups, where the observed rate constant is proportional to the extent of thiol deprotonation at a given pH (2-4). Reduced protein solutions (wild-type and single-cysteine variants) were prepared in 10 mM Mops pH 7 with 2 mM TCEP as reductant. A mixed buffer solution (PCTC buffer: K_2HPO_4, sodium citrate, Tris and CHES, all at 50 mM) was used for each pH experiment. In each case, 2 µl badan (dissolved in DMF) was added to 3 ml PCTC solution giving a final badan concentration of 15 µM. The reaction was then started by addition of 50 µl protein solution to give a final protein concentration ~0.15 µM. The extent of badan modification over time was followed via the increase in badan fluorescence at 510 nm (when excited at 390 nm). Pseudo-first-order rate constants were obtained from fitting the data to Equ. S2:

$$F_t = F_{t=0} + \left(F_{t=\infty} - F_{t=0}\right)e^{-k_o t} \qquad \text{(S2)}$$

where F is the observed fluorescence intensity for a given time point as denoted by subscripts; t = 0 is the start of the experiment, t = ∞ is the endpoint. The observed pseudo-first-order rate constant is denoted k_o and time (in mins) is given as t.

Cysteine pK_a values for each single cysteine variant were determined by fitting a plot of the pseudo-first-order rate constants as a function of pH to a form of the Henderson-Hasselbach equation. For

single protonation/deprotonated events, Equ. S3 was used, while for two independent protonation/deprotonation events, Equ. S4 was used:

$$k_o = k_b + \frac{k_1}{1+10^{pK_{a1}-pH}} \quad \text{(S3)}$$

$$k_o = k_b + \frac{k_1}{1+10^{pK_{a1}-pH}} + \frac{k_2}{1+10^{pK_{a2}-pH}} \quad \text{(S4)}$$

In Equ. S3 and S4, k_o is the pseudo-first-rate constant, k_b is the baseline rate constant, k_1 and k_2 are the rate constants for badan modification of the first and second deprotonated cysteines. In all fits, k_b refined to negligible values.

SUPPLEMENTAL TABLES

Table S1. Oligonucelotides used as primers

Name	Sequence	Restriction site or mutation
LE051	5'- CGGGGTACCGGTGCGGCACAAGCTGAG -3'	
LE052	5'- GTGCAAGCTTGCTCTCAGCTATTCTTCC -3'	*Hin*dIII
LY001	5'- TATGGATCCGGTGCGGCACAAGCTGAG -3'	*Bam*HI
LY002	5'- TCTTGTCGACATGAAAAAAGCTGAGAGTCTATGC -3'	*Sal*I
LY003	5'- CATTTTTGGACGTCATGGGCACCGCCCTGCAAAAAGGAG -3'	C65A
LY004	5'- CTCCTTTTTGCAGGGCGGTGCCCATGACGTCCAAAAATG -3'	C65A
LY005	5'-GACGTCATGGTGCCCGCCCGCCAAAAAGGAGCTTCCACAG-3'	C68A
LY006	5'-CTGTGGAAGCTCCTTTTTGGCGGGCGGGCACCATGACGTC -3'	C68A
LY007	5'- GCCCGCCCTGCAAAAAGCAGCTTCCACAGTTTCAATCG-3'	E71Q
LY008	5'- CGATTGAAACTGTGGAAGCTGCTTTTTGCAGGGCGGGC-3'	E71Q

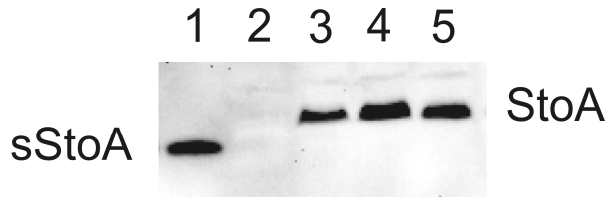

Fig. S1. Western blot analysis for StoA variants.
B. subtilis strains were grown in NSMP with 1 mM IPTG and the cells harvested when the cultures entered stationary growth phase. Proteins in the membrane fraction were separated by SDS-PAGE and probed for StoA antigen. Lane 1, 20 ng of purified sStoA; lane 2, LUL30/pDG148 (negative control); lanes 3-5, LUL30 containing pLYM012, pLYM013 or pLYM015 (encoding E71Q, C68A and C65A variants of StoA, respectively). Approximately 40 μg of membrane protein was loaded in each lane 2-5.

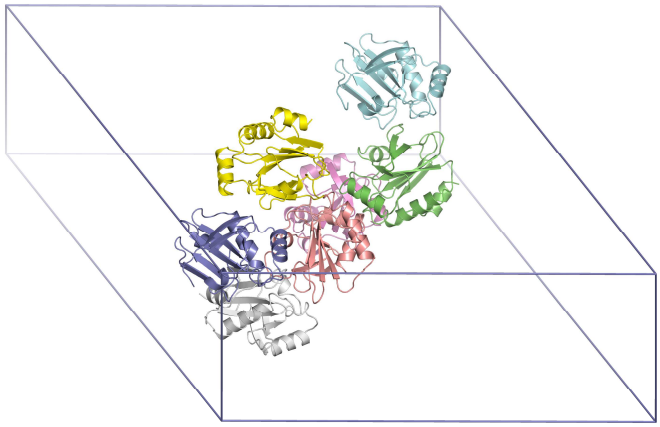

Fig. S2. The asymmetric unit of the sStoA crystals used for structural analysis. The seven sStoA polypeptides found in the asymmetric unit are color coded. See the main paper for details.

SUPPLEMENTARY REFERENCES

1. Hutchinson, R. S., and Ort, D. R. (1995) *Meth. Enzymol.* **252**, 220-228.
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3. Nelson, J. W., and Creighton, T. E. (1994) *Biochemistry* **33**, 5974-5983
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AUTHOR CONTRIBUTIONS

AC conducted the crystallographic work, measured redox potentials, cysteine pK_a values, and pH stabilities. MCM aided AC in initial crystallization trials and produced sStoA protein. YL constructed plasmids, carried out mutagenesis and produced sStoA protein variants and SeMet-labeled protein, conducted Mal-PEG experiments and performed *in vivo* sporulation assays and some of the Western blots. NLB and LH planned and supervised the project. AC, YL, NLB and LH wrote the manuscript.