

Intramolecular Disulfide Bond between Catalytic Cysteines in an Intein Precursor

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Supporting Information

ABSTRACT: Protein splicing is a self-catalyzed and spontaneous post-translational process in which inteins excise themselves out of precursor proteins while the exons are ligated together. We report the first discovery of an intramolecular disulfide bond between the two active-site cysteines, Cys1 and Cys+1, in an intein precursor composed of the hyperthermophilic *Pyrococcus abyssi* PolII intein and extein. The existence of this intramolecular disulfide bond is demonstrated by the effect of reducing agents on the precursor, mutagenesis, and liquid chromatography–mass spectrometry (LC–MS) with tandem MS (MS/MS) of the tryptic peptide containing the intramolecular disulfide bond. The disulfide bond inhibits protein splicing, and splicing can be induced by reducing agents such as tris(2-carboxyethyl)phosphine (TCEP). The stability of the intramolecular disulfide bond is enhanced by electrostatic interactions between the N- and C-exteins but is reduced by elevated temperature. The presence of this intramolecular disulfide bond may contribute to the redox control of splicing activity in hypoxia and at low temperature and point to the intriguing possibility that inteins may act as switches to control extein function.

Protein splicing is a self-catalyzed post-translational process in which an *intervening protein*, called an intein, is excised from a precursor protein, together with the ligation of the two flanking sequences immediately N- and C-terminal to the intein, termed the N- and C-exteins, respectively^{1–3} [Figure S1 in the Supporting Information (SI)]. Protein splicing is strictly intramolecular,⁴ requiring no external cofactor or energy input.^{4,5} Inteins have been found in all domains of life⁶ but exist only in unicellular organisms.⁷ Hedgehog (Hh) proteins, which are crucial for the embryonic patterning of higher eukaryotes, undergo similar autoprocessing in the cholesterolylation of the Hh signaling domain by the Hh processing domain.^{8–10} The Hh processing domain and inteins share many conserved sequence motifs and a common Hh–intein (HINT) fold.¹¹

There is usually a residue with a side-chain nucleophile (e.g., Cys, Ser, Thr) at the junction between the N-extein and the intein (the N-terminal splice junction) and another between the C-extein and the intein (the C-terminal splice junction). These two conserved residues, Cys1 (the first residue of the intein) and Cys+1 (the first residue of the C-extein) for many inteins, serve as the nucleophiles for the first two steps of splicing, N–X acyl shift and transesterification, respectively (Figure S1). Therefore, differential reactivity of these Cys residues can control the steps of protein splicing.^{12,13} Recently, an intramolecular disulfide bond was engineered in an *Ssp* DnaE intein precursor with a CPGC motif,¹⁴ resulting in a stable intein precursor that can be reduced to induce splicing. Likewise, intein-mediated protein ligation was used to create a non-native disulfide bond to trap an intein precursor of the *Methanococcus jannaschii* Klba intein.¹⁵ Salic and co-workers¹⁶ have shown that there is an intramolecular disulfide bond in the Hh processing domain that must be reduced before cholesterolylation can proceed. However, within native intein precursor sequences, intramolecular disulfide bonds between the two catalytic cysteines have not been observed. For Cys1 and Cys+1 to catalyze protein splicing efficiently, the N- and C-terminal splice junctions must be close in space. However, crystal structures of inteins show variable distances between the two junctions, from 3–4 Å up to 8–9 Å.^{17–20}

Recently, we solved the NMR structure of a hyperthermophilic intein interrupting the *Pyrococcus abyssi* DNA polymerase II (*Pab* PolII).^{21,22} The *Pab* PolII intein only splices at high temperature, offering the opportunity to study a stable precursor containing native intein and extein sequences at room temperature. We overexpressed in *Escherichia coli* BL21(DE3) a 25 kDa precursor composed of the *Pab* PolII intein, a short N-extein, and a short His-tagged C-extein that contains Cys1 and Cys+1 as the only two cysteines in the sequence (see the SI). The *Pab* PolII intein precursor was purified by Ni-NTA affinity chromatography. In the SDS-PAGE in Figure 1A, instead of the expected 25 kDa band, a strong 20 kDa band was observed along with a very weak 25 kDa band. We then treated the *Pab* PolII precursor protein with increasing

Received: November 23, 2011

Published: January 23, 2012

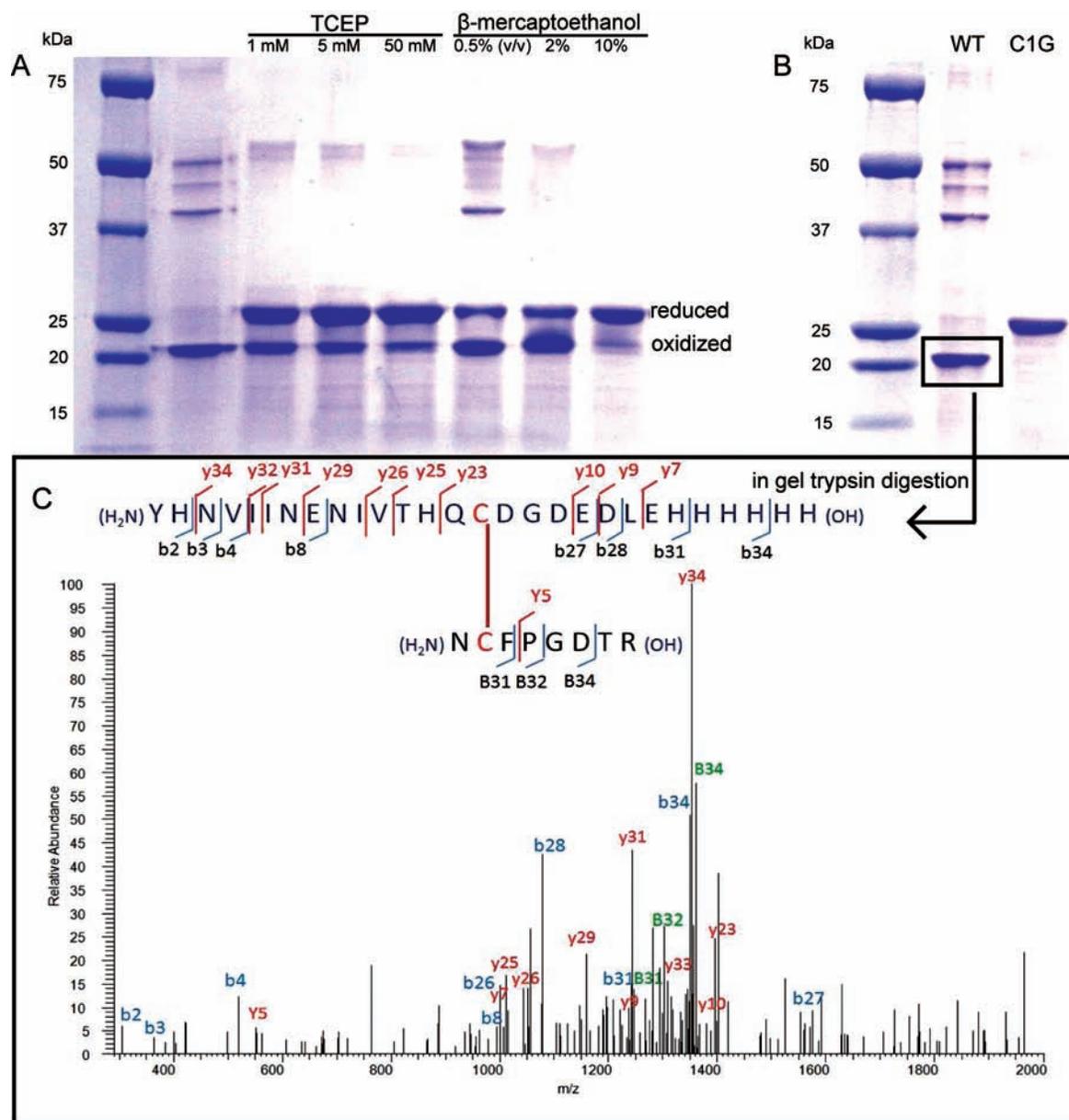


Figure 1. Intramolecular disulfide bond between the two active site cysteines Cys1 and Cys+1. (A) Effect of reducing agents. The *Pab* PolII precursor was treated with 1, 5, or 50 mM TCEP or with 0.5, 2, or 10% (v/v) β -mercaptoethanol. Increasing amounts of reducing agent decreased the intensity of the 20 kDa band while increasing the intensity of the 25 kDa band. (B) Effect of the Cys1Gly mutation, which prevents the formation of an intramolecular disulfide bond. Only the 25 kDa band (reduced precursor) is present for the Cys1Gly precursor. (C) MS/MS identification of the intramolecular disulfide bond. The band in (B) was excised, digested by trypsin, and analyzed by LC-MS/MS. Full sequence coverage was achieved (see the SI). A peptide containing the intramolecular disulfide bond was detected and identified by accurate MS and MS/MS in the digestion mixture, demonstrating that the *Pab* PolII precursor forms an intramolecular disulfide bond.

amounts of a reducing agent [tris(2-carboxyethyl)phosphine (TCEP) or β -mercaptoethanol] at room temperature, which is below the optimal temperature for protein splicing of this intein. The 25 kDa band became progressively stronger, with a concomitant decrease in the 20 kDa band. We suspected that an intramolecular disulfide bond was formed between the two catalytic cysteine residues, resulting in a circle-like protein which increased the rate of migration in nonreducing SDS-PAGE.²³ Therefore, the 20 kDa protein band likely was due to the disulfide-linked form of the intein precursor (oxidized precursor), which was then converted to the expected 25 kDa band (reduced precursor) upon reduction at a temperature that does not permit splicing. Additional bands observed by SDS-

PAGE between 40 and 50 kDa likely were due to intein dimers, as they disappeared with the addition of reducing agent. There are two cysteines in each intein monomer, and therefore, several different types of intermolecular disulfide bonds could form, giving rise to multiple bands.

The identification of the 20 kDa band as an intein with an intramolecular disulfide bond was further supported by the comparison with a Cys1Gly mutant, which cannot form an intramolecular disulfide bond. As expected, the 20 kDa band was absent for this mutant (Figure 1B). The wild-type (WT) precursor protein migrating at 20 kDa was then excised from the SDS-PAGE, digested by trypsin, and analyzed by LC-MS/MS. All of the predicted tryptic peptides were identified except

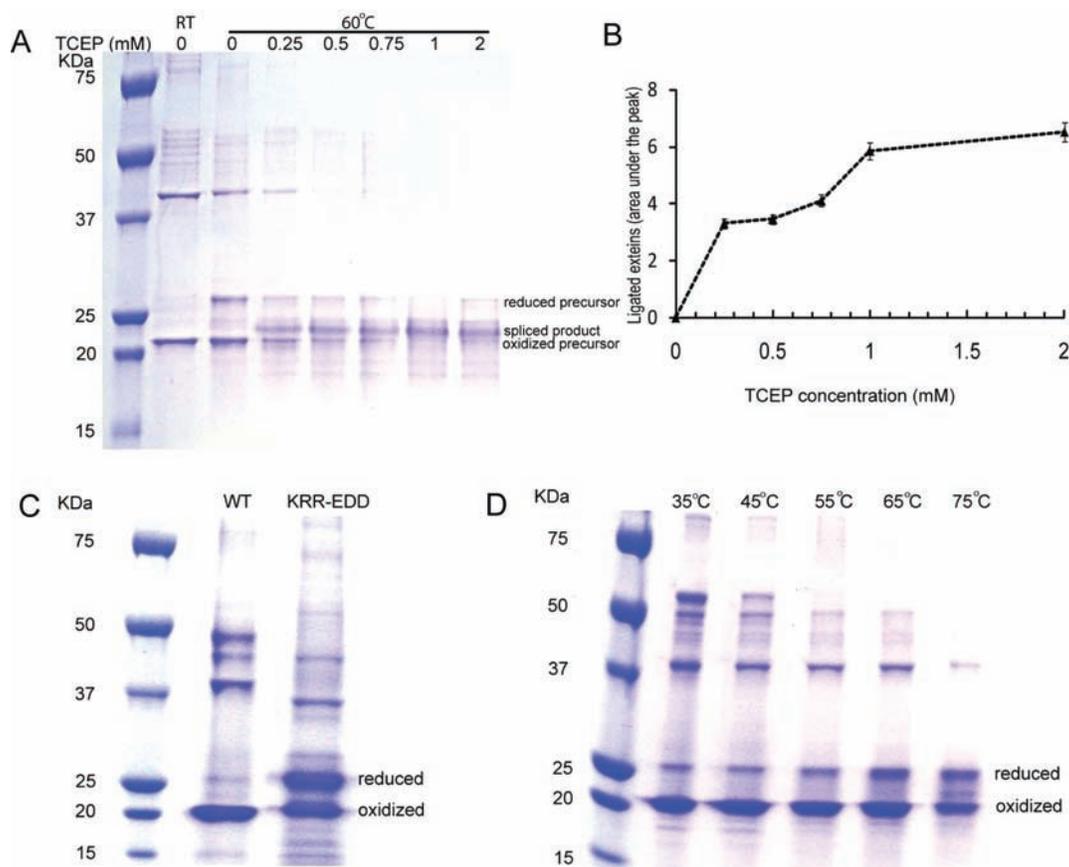


Figure 2. (A) Precursor splicing with and without TCEP. The precursor protein was treated with 0.25, 0.5, 0.75, 1, and 2 mM TCEP at 60 °C overnight for splicing. (B) Greater amounts of spliced extein were detected by MS/MS with increasing amounts of TCEP. (C) Comparison between the WT and the KRR-EDD mutant showing the effect of charge–charge interactions in exteins on disulfide bond formation. (D) Temperature dependence of the intramolecular disulfide bond, with higher temperature favoring the reduced species. *Pab* PolII intein precursor was incubated at 35, 45, 55, 65, and 75 °C overnight.

for the ones containing a single cysteine (Figure S2). Instead, a tryptic peptide containing covalently linked Cys1 and Cys+1 was detected and identified by accurate MS and MS/MS, conclusively demonstrating the presence of the intramolecular disulfide bond between the two active-site cysteines (Figure 1C). This disulfide in the *Pab* PolII intein precursor is particularly strong, as it forms upon overexpression in *E. coli* BL21 (DE3) rather than requiring *trxB* and *gor* strains.

Next, we tested whether the presence of the intramolecular disulfide affects the splicing activity (Figure 2A,B). Very little ligated extein was detected after overnight incubation at 60 °C in the absence of reducing agent, indicating that the intramolecular disulfide bond inhibits splicing. TCEP was then introduced to break the disulfide bond to initiate splicing. Ligated exteins, detected by LC/MS and confirmed by MS/MS, were generated in the presence of 0.25 mM TCEP (Figures S3 and S4). Spliced exteins accumulated with increasing TCEP concentration up to 2 mM (Figure 2A,B).

Next, we explored the influence of extein residues on the formation of the disulfide bond. In this precursor, three of the four C-terminal residues of the N-extein are positively charged and three of the four N-terminal residues of the C-extein are negatively charged (see the SI). This might provide attractive forces that favor disulfide bond formation. Mutation of Lys(−4), Arg(−3), and Arg(−2) in the N-extein to Glu, Asp, and Asp, respectively, resulted in a significant decrease in the oxidized species with a concurrent increase in the reduced

species in SDS-PAGE (Figure 2C). This suggests that electrostatic interactions between the N- and C-exteins play an important role in the formation of the intramolecular disulfide bond and in coordinating the N- and C-terminal splice junction. Electrostatic interactions were also observed to assist in the reassociation of the fragments of the split *Npu* DnaE intein.²⁴

We tested whether temperature can change the equilibrium between the reduced and oxidized forms of the precursor. Purified *Pab* PolII intein precursor protein was incubated at 35, 45, 55, 65, and 75 °C overnight at pH 6.5 (Figure 2D). The protein aggregated at temperatures above 80 °C (data not shown). With increased temperature, there was an increased amount of reduced precursor and a decreased amount of oxidized precursor, indicating that the disulfide bond is weakened by high temperature, which may help account for the temperature dependence of the splicing activity.

This is the first time that an intramolecular disulfide bond between the two active-site cysteines Cys1 and Cys+1 has been discovered in a native intein precursor protein. The two cysteines are separated by 185 residues of primary sequence, in contrast to engineered intein disulfide bonds separated by just two residues, such as that formed by the CPGC motif.¹⁴ In the Hh processing domain, Cys1 forms a disulfide bridge with another cysteine that is *not* homologous to Cys+1, and this bridge must be reduced by a protein disulfide isomerase before cholesteroylation.¹⁶ Salic and co-workers proposed that the

disulfide may be important for the folding of the Hh processing domain,¹⁶ but this is not the case for the *Pab* PolII intein, which has a well-folded structure without the intramolecular disulfide bond.²²

The *Pab* PolII intein interrupts a crucial extein, the DNA polymerase II DP2 subunit, which is essential for DNA replication in *Pab*.^{25,26} *Pab* is anaerobic, and the presence of oxygen may impose oxidative stress and result in a more oxidizing cellular environment. Under these conditions, disulfide formation between Cys1 and Cys+1 may be promoted, inhibiting splicing and the formation of the fused active exteins, the DNA PolII DP2 subunit. This may arrest DNA replication to preserve the integrity of the genome during oxidative stress. Callahan et al.¹⁴ have proposed a similar redox switch for the intein that interrupts the *Pab* MoaA. The molybdopterin cofactor produced by active MoaA is used in a variety of redox-dependent enzymes. Although disulfide bonds are unusual in intracellular proteins, genomic studies suggest that they are more common in hyperthermophilic archaeobacteria, including *Pab*, suggesting that the intramolecular disulfide bond might be relevant in vivo.^{27,28}

The temperature-dependence of the *Pab* PolII intein may also be a function of the redox switch and possibly play a role in regulation of extein activity. Below its optimal growth temperature of ~100 °C, *Pab* must shut down DNA replication. As shown in Figure 2D, more intramolecular disulfide bond is present at lower temperatures for the *Pab* PolII precursor, inhibiting splicing. With native exteins, this would prevent the formation of active DNA polymerase DP2. The redox sensitivity of this intramolecular disulfide bond may contribute to the mechanism by which *Pab* stops replication, even at relatively moderate temperatures such as 37 °C.

Interestingly, the intein interrupting another essential DNA replication protein in *Pab*, replication factor 2,²⁹ also has the conserved residues Cys1 and Cys+1. Although the intein has yet to be studied, it may further contribute to redox regulation in the physiological response to hyperoxia and low temperature in *Pab* using redox chemistry of the intramolecular disulfide bond. It is intriguing that inteins may not be just parasitic elements in protein or DNA sequences;⁷ instead, inteins may act as switches and regulate the function of crucial exteins and cellular physiology through redox sensitivity conferred by intramolecular disulfide bonds.

In summary, for the first time we have conclusively established the existence of an intramolecular disulfide bond between two active-site residues, Cys1 and Cys+1, in an intein precursor composed of native *Pab* PolII intein and exteins. Our findings suggest that the redox chemistry of the intramolecular disulfide bond may regulate protein splicing and extein function.

■ ASSOCIATED CONTENT

Supporting Information

Detailed materials and methods, illustration of the four steps of protein splicing, and additional LC–MS and MS/MS data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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■ ACKNOWLEDGMENTS

This work was supported by NIH (Grant GM081408 to C.W.) and NSF (Grant 0950245 to K.V.M.).

■ REFERENCES

- (1) Hirata, R.; Ohsumk, Y.; Nakano, A.; Kawasaki, H.; Suzuki, K.; Anraku, Y. *J. Biol. Chem.* **1990**, *265*, 6726.
- (2) Kane, P. M.; Yamashiro, C. T.; Wolczyk, D. F.; Neff, N.; Goebel, M.; Stevens, T. H. *Science* **1990**, *250*, 651.
- (3) Cooper, A. A.; Stevens, T. H. *Trends Biochem. Sci.* **1995**, *20*, 351.
- (4) Kawasaki, M.; Satow, Y.; Ohya, Y.; Anraku, Y. *FEBS Lett.* **1997**, *412*, 518.
- (5) Xu, M. Q.; Southworth, M. W.; Mersha, F. B.; Hornstra, L. J.; Perler, F. B. *Cell* **1993**, *75*, 1371.
- (6) Perler, F. B. *Nucleic Acids Res.* **2002**, *30*, 383.
- (7) Pietrovski, S. *Trends Genet.* **2001**, *17*, 465.
- (8) Jeong, J.; McMahan, A. P. *J. Clin. Invest.* **2002**, *110*, 591.
- (9) Porter, J. A.; von Kessler, D. P.; Ekker, S. C.; Young, K. E.; Lee, J. J.; Moses, K.; Beachy, P. A. *Nature* **1995**, *374*, 363.
- (10) Porter, J. A.; Young, K. E.; Beachy, P. A. *Science* **1996**, *274*, 255.
- (11) Hall, T. M.; Porter, J. A.; Young, K. E.; Koonin, E. V.; Beachy, P. A.; Leahy, D. J. *Cell* **1997**, *91*, 85.
- (12) Shingledecker, K.; Jiang, S.; Paulus, H. *Arch. Biochem. Biophys.* **2000**, *375*, 138.
- (13) Du, Z.; Zheng, Y.; Patterson, M.; Liu, Y.; Wang, C. *J. Am. Chem. Soc.* **2011**, *133*, 10275.
- (14) Callahan, B. P.; Topilina, N. I.; Stanger, M. J.; Van Roey, P.; Belfort, M. *Nat. Struct. Mol. Biol.* **2011**, *18*, 630.
- (15) Saleh, L.; Southworth, M. W.; Considine, N.; O'Neill, C.; Benner, J.; Bollinger, J. M.; Perler, F. B. *Biochemistry* **2011**, *50*, 10576.
- (16) Chen, X.; Tukachinsky, H.; Huang, C. H.; Jao, C.; Chu, Y. R.; Tang, H. Y.; Mueller, B.; Schulman, S.; Rapoport, T. A.; Salic, A. *J. Cell Biol.* **2011**, *192*, 825.
- (17) Ding, Y.; Xu, M. Q.; Ghosh, I.; Chen, X.; Ferrandon, S.; Lesage, G.; Rao, Z. *J. Biol. Chem.* **2003**, *278*, 39133.
- (18) Duan, X.; Gimble, F. S.; Quijcho, F. A. *Cell* **1997**, *89*, 555.
- (19) Mizutani, R.; Nogami, S.; Kawasaki, M.; Ohya, Y.; Anraku, Y.; Satow, Y. *J. Mol. Biol.* **2002**, *316*, 919.
- (20) Poland, B. W.; Xu, M. Q.; Quijcho, F. A. *J. Biol. Chem.* **2000**, *275*, 16408.
- (21) Liu, J.; Du, Z.; Albracht, C. D.; Naidu, R. O.; Mills, K. V.; Wang, C. *Biomol. NMR Assignments* **2011**, *5*, 233.
- (22) Du, Z.; Liu, J.; Albracht, C. D.; Hsu, A.; Chen, W.; Marieni, M. D.; Colelli, K. M.; Williams, J. E.; Reitter, J. N.; Mills, K. V.; Wang, C. *J. Biol. Chem.* **2011**, *286*, 38638.
- (23) Powers, J. L.; Andrews, C. S.; St. Antoine, C. C.; Jain, S. S.; Bevilacqua, V. L. H. *J. Chem. Educ.* **2005**, *82*, 93.
- (24) Shah, N. H.; Vila-Perello, M.; Muir, T. W. *Angew. Chem., Int. Ed.* **2011**, *50*, 6511.
- (25) Cohen, G. N.; Barbe, V.; Flament, D.; Galperin, M.; Heilig, R.; Lecompte, O.; Poch, O.; Prieur, D.; Quérellou, J.; Ripp, R.; Thierry, J.-C.; Van der Oost, J.; Weissenbach, J.; Zivanovic, Y.; Forterre, P. *Mol. Microbiol.* **2003**, *47*, 1495.
- (26) Henneke, G.; Flament, D.; Hübscher, U.; Querellou, J.; Raffin, J.-P. *J. Mol. Biol.* **2005**, *350*, 53.
- (27) Mallick, P.; Boutz, D. R.; Eisenberg, D.; Yeates, T. O. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 9679.
- (28) Ladenstein, R.; Ren, B. *Extremophiles* **2008**, *12*, 29.
- (29) Henneke, G.; Gueguen, Y.; Flament, D.; Azam, P.; Querellou, J.; Dietrich, J.; Hübscher, U.; Raffin, J.-P. *J. Mol. Biol.* **2002**, *323*, 795.