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LETTER TO THE EDITOR

Structural insights into DndE from *Escherichia coli* B7A involved in DNA phosphorothioation modification

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Dear Editor,

DNA phosphorothioate (PT) modification, originally developed as an artificial tool to stabilize oligodeoxynucleotides against nuclease degradation [1], was recently found to be incorporated with sulfur into DNA backbone as a novel physiological variation by the five-gene *dnd* cluster (*dndA-dndE*) products in a sequence- and stereo-specific manner [2]. This PT modification causes the DNA degradation (Dnd) phenotype and is widespread and quantized in bacterial genomes, working as a part of a restriction modification system [3-5]. This modification can be specifically cleaved *in vitro* by type IV restriction endonuclease [6]. DndA works as a cysteine desulfurase and assembles DndC as a 4Fe-4S cluster protein [7]. DndC possesses ATP pyrophosphatase activity [8, 9] and is predicted to have 3′-phosphoadenosine-5′-phosphosulfate (PAPS) reductase activity, whereas DndB has homology to a group of transcriptional regulators [10, 11]. DndD, known as SpfD in *Pseudomonas fluorescens* Pf0-1, has ATPase activity possibly related to DNA structure alteration or nicking during PT incorporation [5, 12]. Sequence identity (46%) and similarity (61%) to phosphoribosylaminoimidazole carboxylase (NCAIR synthetase) from *Anabaena variabilis* [11] suggest that DndE could be a NCAIR synthase analogue [13]. However, DndE may also act as a sulfotransferase due to a specific PAPS binding sequence AAVGK-TLLIHLHR contained in the C-terminus of DndE from *Streptomyces lividans* ($DndE_{strep}$) [10]. Therefore, the exact function of DndE remains unknown.

To address this, we over-expressed two DndE proteins from *Escherichia Coli* B7A ($DndE_{B7A\text{-}full}$) and *Salmonella enterica* ($DndE_{Salm\text{-}full}$), respectively (Supplementary information, Data S1), both having isoelectric point values close to 9.5. Sequence alignment indicates that they have high similarity except for several residues in their C-termini (Figure 1A). The sequence-selectivity of PT modification in both strains is a d(G_{PS}A) or a d(G_{PS}T) dinucleotide with an R_P PT bond [5]. $DndE_{B7A\text{-}full}$ is unstable, so we used its C-terminus-truncated form (aa 1-110, $DndE_{B7A\text{-}N110}$) for crystallization. The aggregation states of DndE proteins were determined by dynamic light scattering (DLS) assay (Supplementary information, Data S1), indicating the formation of tetramers at the concentration of 200 μM. Two-dimensional NMR ^{1}H-^{15}N HSQC spectra on ^{15}N-labeled $DndE_{B7A\text{-}N110}$ suggest that the $DndE_{B7A\text{-}N110}$ fragment in Tris buffer is more homogeneous (Supplementary information, Figure S1). The structure was determined using the single-wavelength anomalous dispersion technique, and refined to 2.5 Å resolution with an R_{free} factor of 24.2% and an R factor of 20.1% (Supplementary information, Table S1).

The $DndE_{B7A\text{-}N110}$ structure reveals a tetramer conformer (Figure 1B), consistent with the results from DLS assay. The tetramer adopts a quadrate fold, resembling a four-leaf clover of 62 Å in both length and width and 32 Å in height. It contains two kinds of dimers (A-E and A-M) with different stability resulted from different numbers of H-bonds in the dimer interface (Figure 1C and 1D). Two clefts (~60° and ~90°) are formed by the dimers (Figure 1E and 1F). Electrostatic surface analysis indicates that the cleft-containing side is more hydrophobic than its opposite side (Figure 1G and 1H). The K20 side-chain in each monomer extends into the tetramer center, forming hydrogen bonds with G24 and/or G21 in the next monomer, producing a large positively charged hole (Figure 1B-1D and 1G). The four monomer structures are almost identical and can be superimposed with an root-mean-square deviation of 0.33 Å over 59 backbone Cα atoms in the secondary structural region. Each monomer comprises five helices: H1 (aa 10-22), H2 (aa 27-39), H3 (aa 62-66), H4 (aa 70-77) and H5 (aa 86-102) (Figure 1A). Together with the H2 helix, a long flexible loop (aa 42-60) between the H2 and H3 helices encloses the H5 helix, where the residues R49, D50, S51 and K52 are not observable in some monomers.

Dali search indicates that $DndE_{B7A\text{-}N110}$ is a novel DNA-binding protein (Supplementary information, Figure S2). Thus, we performed $DndE_{B7A\text{-}N110}$ binding studies using different DNA substrates *in vitro*, including dsDNA with or without a specific sequence (5′-GAAC-3′) for

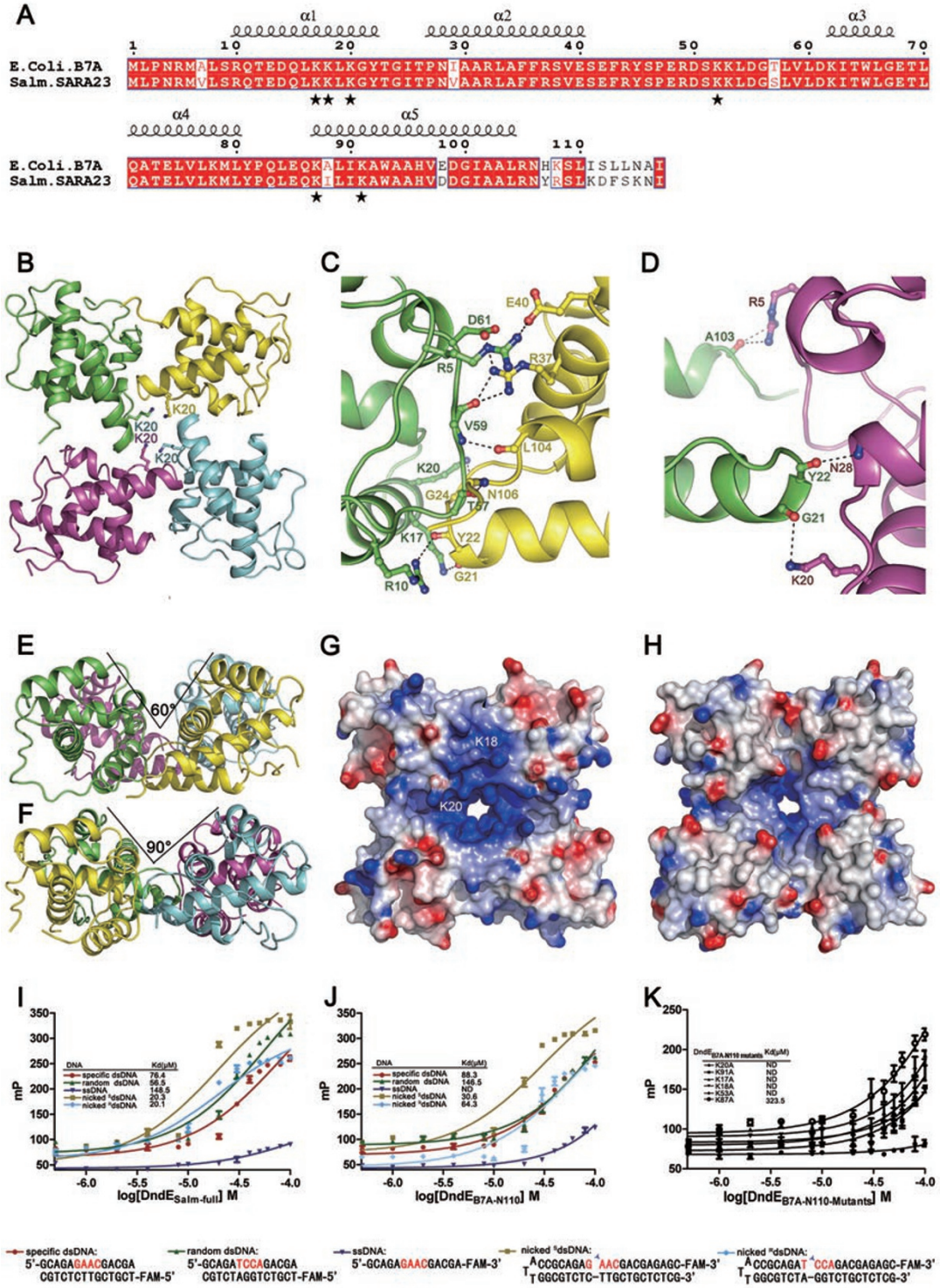

Figure 1 DndE structure analysis and fluorescence polarization assay for DNA-binding measurement. **(A)** Sequence alignment of $DndE_{B7A\text{-}full}$ and $DndE_{Salm\text{-}full}$ with structural elements labeled on the top. **(B)** Ribbon diagram of $DndE_{B7A\text{-}N110}$ tetramer, monomer A is shown in green, E in purple, C in royal blue and M in yellow. **(C**, **D)** Interfaces between A-M monomers and between A-E monomers. **(E)** Cleft formed in $DndE_{B7A\text{-}N110}$ tetramer. **(F)** The view rotated by 90° relative to the x axis from **E**. **(G)** Electrostatic surface representation of $DndE_{B7A\text{-}N110}$ tetramer at the same orientation with **B**. **(H)** The view rotated by 90° relative to the x axis from **G**. **(I-K)** Fluorescence polarization assay for DNA interaction with **(I)** $DndE_{Salm\text{-}full}$, **(J)** $DndE_{B7A\text{-}N110}$ and **(K)** $DndE_{B7A\text{-}N110}$ mutants, respectively. In the bottom, the sequences of DNA substrates were indicated, where the blur star represents phosphate.

PT modification as found in *E. coli* B7A and *Salmonella* (specific dsDNA and random dsDNA in Figure 1). Since DndD is an ATPase with putative DNA-nicking actitvity [5, 12], we assumed that, before PT modification, DndD might hydrolyze the P-O bond between the G-A bases in PT modification sequence. Thus, two nicked dsDNA species were also tested (in one sequence, nicking is located between G-A bases in a specific sequence for PT modification, named as nicked ^{S}dsDNA; in the other sequence, nicking is designed between T-C bases in a random sequence, denoted as nicked ^{R}dsDNA, as shown in Figure 1), as well as a sequence-specific single-stranded DNA (ssDNA in Figure 1). Among different DNA substrates, $DndE_{Salm\text{-}full}$ has the strongest binding affinity to nicked dsDNA (for both nicked ^{S}dsDNA and nicked ^{R}dsDNA, K_D ~20 μM), indicating that DndE might be a nicked dsDNA binding protein (Figure 1I), and that the specific –GAAC–sequence might not be relevant for nicked DNA binding. $DndE_{B7A\text{-}N110}$ has a higher binding affinity to nicked ^{S}dsDNA (K_D ~30 μM) than that to nicked ^{R}dsDNA (K_D ~60 μM), specific dsDNA (K_D ~90 μM), random dsDNA (K_D ~146.5 μM), and specific ssDNA (binding is non-detectable) (Figure 1J, Supplementary information, Table S2), suggesting that $DndE_{B7A\text{-}N110}$ also slightly prefers to bind with nicked dsDNA. Among the mutants where positively charged residues in the $DndE_{B7A\text{-}N110}$ surface including K17, K18, K20, K53, K87 and K91 are replaced by alanine, the K20A and K18A mutants showed significant decreases in binding affinities to specific dsDNA (K20A, K_D ~690 μM; K18A, non-detectable). All these variants showed much weaker (K87A, K_D 323 μM, decreased by 10-fold) or non-detectable binding to nicked ^{S}dsDNA (Figure 1K), further supporting that all these positively charged residues might be important for interaction with nicked dsDNA.

In addition, we measured the interaction of $DndE_{B7A\text{-}N110}$ with PAPS, ATP or ADP using the isothermal titration calorimetry assay (Supplementary information, Figure S3). In contrast with the early prediction [10-11, 13], $DndE_{B7A\text{-}N110}$ showed no binding affinities to any of them (Supplementary information, Figure S3), consistent with the fact that the $DndE_{B7A\text{-}N110}$ structure includes neither a 5′-PSB motif nor a 3′-PB motif for PAPS and PAP binding [14], nor a glycine-rich P loop for ATP or ADP phosphate binding as seen in the NCAIR synthetase [15, 16].

Taken together, the crystal structure of $DndE_{B7A\text{-}N110}$ reveals that DndE is neither a sulfotransferase nor a NCAIR synthase analogue, but a possible nicked dsDNA binding protein with a previously unrecognized fold. Thus, DNA nicking (probably by DndD [5, 12]) and nicked DNA binding by DndE might be essential for DNA PT modification.

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Wei Hu[1, *], Chengkun Wang[1, *], Jingdan Liang[2], Tianlong Zhang[3], Zhongpei Hu[1, 4], Zhijun Wang[2], Wenxian Lan[1], Fang Li[4], Houming Wu[1], Jianping Ding[3], Geng Wu[2], Zixin Deng[2], Chunyang Cao[1]

[1]*State Key Laboratory of Bio-organic and Natural Product Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, 345 Lingling Road, Shanghai 200032, China;* [2]*Laboratory of Microbial Metabolism, and School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200030, China;* [3]*Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Shanghai 200031, China;* [4]*Central China Normal University, Wuhan, Hubei 430079, China*

*These two authors contributed equally to this work.
Correspondence: Chunyang Cao
Tel: 86-21-54925491; Fax: 86-21-64166128
E-mail: ccao@mail.sioc.ac.cn

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)