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The central loop of *Escherichia coli* glutamine synthetase is flexible and functionally passive ☆

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Abstract

Bacterial glutamine synthetases (GSs) are dodecameric aggregates comprised of two face-to-face hexameric rings, which form a cylindrical aqueous channel. Available crystal structures indicate that each subunit provides a 'central loop' that protrudes into this channel. Residues on either side of this loop contribute directly to substrate or metal ion cofactor binding. Although it has been suggested that this conspicuous structural feature may be functionally important, a systematic structure–function analysis of this loop has not been done. Here, we examine the behavior of a cysteine mutant, E165C, which yields inter-subunit disulfide bonds connecting the central loops. The inter-subunit disulfide bonds are readily detected by electrospray ionization mass spectrometry. Based on molecular models, the disulfide bonds would form only if the engineered cysteines on adjacent subunits moved ~5 Å. Surprisingly, inter-subunit disulfide bonds between the central loops caused no detectable changes in the K_Ms for glutamate or ATP, nor the K_D for either ATP or the transition state analog (L)-methionine sulfoximine (MSOX). Furthermore, covalent and quantitative adduction of the E165C mutant with iodo-acetamido-pyrene yielded nearly fully active enzyme bearing fluorescent pyrene excimers. The relative contribution of pyrene monomers to excimers in the steady state fluorescence is temperature dependent, suggesting thermal equilibrium between loop conformational states. However, the monomer–excimer ratio is independent of ligands such as MSOX, glutamate, or Mn^{2+}. These results validate the suspected flexibility of the central loop, but raise significant doubt about its direct functional role in GS catalysis via conformational switching, including the proposed regulation of GS via ADP-ribosylation within this loop.

Keywords: Conformational dynamics; Protein engineering; Pyrene fluorescence; Exo-site; Tuberculosis

The bacterial glutamine synthetases (GSs) are dodecameric aggregates of identical subunits, formed from two face-to-face hexameric rings ([1–3]; Fig. 1). GSs catalyze the ATP[1]-dependent condensation of ammonia with glutamate to form glutamine, ADP, and HPO_3^-. The chemical and kinetic mechanisms are established, and detailed structure–function analyses have been performed, including catalytic functions for specific enzyme residues [4–10]. The enzyme from *Escherichia coli* has served as a model for complex regulation of nitrogen metabolism, wherein feedback inhibition is mediated by several downstream metabolites including alanine, CTP, glycine, and serine [11]. The enzyme is regulated also by covalent adenylylation of a Tyr in each subunit, on the exterior surface of the ring structure. The adenylylation process includes, in turn, a highly regulated bicyclic cascade enzyme system that is responsive to environmental nitrogen levels [12–14].

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[1] *Abbreviations used:* E165C, the site-directed mutant containing cysteine at position 165; E165C–pyrene, the site-directed mutant adducted with iodo-acetamido-pyrene; amu, atomic mass units; ATP, adenosine-5′-triphosphate; Da, Daltons; glu, glutamate; gln, glutamine; HIV, human immunodeficiency virus; Hepes, (*N*-[2-hydroxyethyl])-piperazine-*N*′-[2-ethane sulfonic acid]; PIA, iodo-acetamido-pyrene; PIPEs, piperazine-*N*-*N*′-bis-(2-ethane sulfonic acid); TCEP, tris-(2-carboxyethyl)phosphine; TFA, trifluoroacetic acid.

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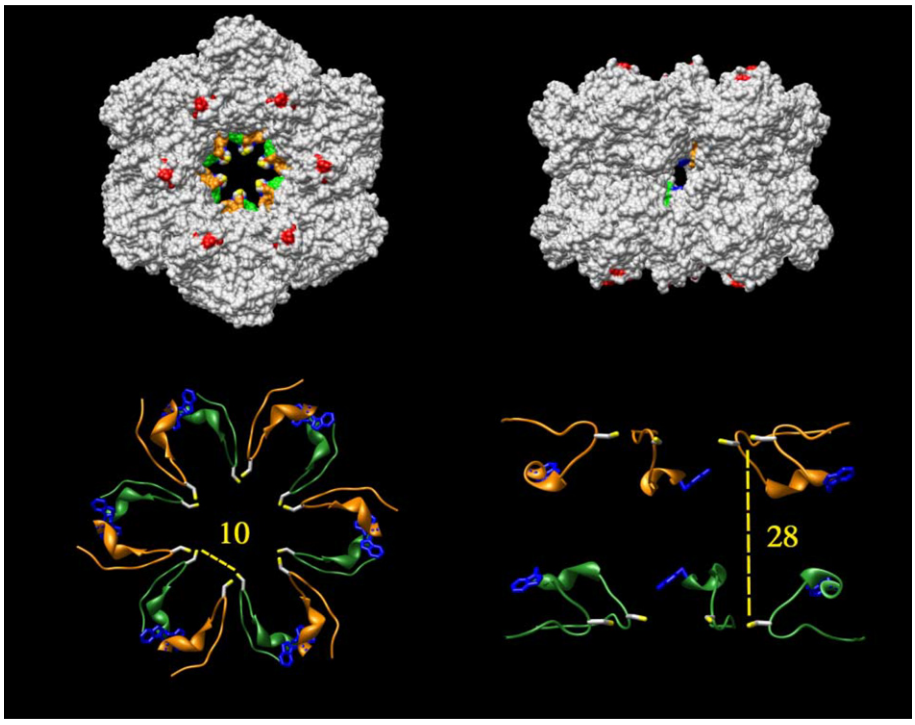

Fig. 1. Structure of *S. typhimurium* glutamine synthetase. The dodecamer (top, space filling model) consists of two face-to-face hexagonal rings of identical subunits. On the left, the sixfold axis of symmetry is perpendicular to the page. On the right, the sixfold axis is vertical, within the page. At the center of the dodecamer, the 'central loop' of each subunit (residues 158–172) is colored orange in the top hexameric ring and/or green in the bottom hexameric ring. Within each loop, the white atoms are from an engineered Cys-165, with yellow representing the sulfur atom. Also shown are the Met-8 backbone atoms (red) on each subunit. The M8C mutant was also used in this study as described in the text. In the bottom structures, the central loops are shown, in the same orientation as in the top structures, from within the central cavity. Because the perspective is from within the central cavity, only 8 of 12 loops are visible in the bottom right panel. The additional blue residue evident in this close up is Trp-158 which is at the base of central loop and which contributes to the active site. The distances between sulfur atoms of the engineered Cys-165 within a hexameric ring are 10.5 Å, and the shortest inter-ring Cys to Cys distances are 28 Å. Thus, intra-ring disulfide bonds are more likely to form than inter-ring disulfide bonds.

Recently, GS from *Mycobacterium tuberculosis* (Mtb) was found to be excreted into the extracellular milieu, and GS inhibitors were cytostatic when added to cultures [15–17]. Moreover, in a guinea pig tuberculosis model, GS was essential for virulence [18]. In short, the *glnA1* gene encoding GS appears to be essential for Mtb survival and pathogenicity [19,20]. As a result, GS has become a potential target for tuberculosis therapy, and pharmacological interference with inhibitors of Mtb GS may be beneficial [16–18,21]. In principle, further characterization of the structure and function of bacterial GSs could facilitate design and optimization of Mtb-specific inhibitors.

The crystal structure of the Mtb GS suggests significant flexibility in functionally important loops [22]. An interesting structural feature of several GSs, that was evident even in the initial low resolution structure [2], is the 'central loop.' The central loop, residues 156–173 of the *E. coli* GS, protrudes into the central aqueous cavity (Fig. 1). Toward the N-terminus of the central loop, residues Glu-129 and Glu-131 are functionally important active site residues and they undergo conformational rearrangement upon binding of metal ion, glutamate or the transition state analog L-methionine sulfoximine [MSOX; 1,22]. To the C-terminus of the central loop, Tyr-179 and Lys-176 contribute to the ammonium binding site and glutamate binding, respectively [1,22]. Proteolytic cleavage of the central loop causes inactivation, although the gross dodecameric structure remains intact [23,24]. In addition, the central loop has been suggested to be the target of ADP-ribosylation for several isoforms including *E. coli* GS [25–27], which may provide a further mechanism of regulation. Based on these observations and suggestions, together with its striking entry into the central channel, we have sought to determine in greater detail the function and properties of the central loop. As expected on the basis of crystallographic analysis, we report here that central loop is indeed highly flexible at its most solvent accessible point. However, we find no evidence of any functional role of loop motion, nor communication between the distal point of the loop and active site residues that contribute to steady state kinetic behavior. This prompts skepticism about