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Research paper

Insight into the structural flexibility and function of *Mycobacterium tuberculosis* isocitrate lyase

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ABSTRACT

Isocitrate lyase (ICL), is a key enzyme of the glyoxylate shunt crucial for the survival of *Mycobacterium tuberculosis* (Mtb) in macrophages during persistent infection. MtbICL catalyses the first step of this carbon anaplerosis cycle and is considered as a potential anti-tubercular drug target. The MtbICL is a tetramer with 222 symmetry, and each subunit of the enzymeis composed of 14 α-helices and 14 β-strands. We studied the conformational flexibility of the enzyme to get a deeper insight into its stability and function. Our studies show that the mutation of His180, close to the MtbICL signature sequence (K_{193}KCGH_{197}) completely abolishes the oligomeric conformation and function of the enzyme. Molecular dynamics studies suggest that the loss of interaction between His180 and Tyr89 most likely alters the orientation of Tyr89 side chain, thereby causing the movement of helices α6, α12, α13 and α14 in the vicinity and affecting the tetrameric assembly. We further show that the oligomerization of MtbICL is primarily mediated by the inter subunit interactions, and strengthened by the helix swapping of α12 −α13 between adjacent subunits. Furthermore, the enzyme activity is influenced by the interactions between the residues of lid region (P_{411}NSSTTALTGSTEEGQFH_{428}) and the loop region (T_{391}KHQREV$_{397}$). Mutation of glutamates of the lid region to non homologous residues (E423A or E424A) or basic residues (E423K or E424K) inactivates the enzyme, whereas the activity is not much compromised in case of homologous mutations (E423D or E424D).

1. Introduction

Tuberculosis (TB) remains an expanding global health crisis responsible for about 2 million deaths annually [1,2]. Nearly about one-third of the world's population harbors latent *Mycobacterium tuberculosis* (Mtb) and would have a positive skin test for the infection [1–4]. There is no effective vaccine against the infection and the prolonged therapy frequently leads to the emergence of multi-drug resistance not only to the first line, but also to some of the second-line drugs [1–5]. The extended therapy is needed to tackle the non replicating dormant population of Mtb in patient lesions which are refractory to killing by the currently used anti-TB drugs, which are capable of killing only the actively dividing bacilli [6]. Thus combating TB requires new therapeutic strategies which can effectively target the dormant bacilli and act in synergy with the available frontline drugs [1]. Hence, the hunt for novel, safer TB drugs effective in treating drug resistant as well as persistent infection is still on.

Many recent works are beginning to elucidate the metabolic adaptation by Mtb during persistence, for instance, the dependence on purine, amino acid and pantothenate biosynthesis, iron acquisition, glyoxylate shunt etc [7]. Beta-oxidation, gluconeogenesis and glyoxylate shunt are important for the survival of Mtb inside the phagosomes of macrophages, which are glucose deficient, but fatty acid replete [8]. Isocitrate lyase (ICL), one of the key enzymes of the glyoxylate shunt catalyses the conversion of isocitrate to succinate and glyoxylate. The enzyme is essential for carbon anaplerosis in the TCA cycle during growth on C2 substrates such as fatty acids [9,10]. The glyoxylate shunt is absent invertebrates, but are widespread among prokaryotes, lower eukaryotes and plants

Abbreviation: Mtb, *Mycobacterium tuberculosis*; ICL, isocitrate lyase; MtbICL, *Mtuberculosis* isocitrate lyase; SEC, size-exclusion chromatography.

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[11]. The expression of ICL is upregulated during infection of macrophages by *Mycobacterium sp*. and the disruption of ICL gene inhibits the persistence of Mtb in macrophage and in mice [12–14]. Hence ICL of Mtb (MtbICL) is considered as one of the potential and attractive drug targets against persistent infection.

The crystal structures of MtbICL in apo form as well as in complex with inhibitors are solved [15]. MtbICL (428 amino acids) is a tetramer with 222 symmetry (Fig. 1). Each subunit of MtbICL is composed of 14 α-helices and 14 β-strands. The core of the structure is an unusual α/β-barrel composed of eight α-helices (α4–α11) and eight β-strands (β2–β5, β8, β12–β14) forming the larger domain of the enzyme. Helix α12 (residues 349–367) projects away from the barrel and together with the two consequent helices α13 (residues 370–384) and α14 (residues 399–409), forms interactions exclusively with the neighboring subunits. A small β-domain consisting of a short five stranded β-sheet (β6, β7, β9, β10, β11) lies atop of the α/β barrel and contains several of the active site residues. The striking feature of this structure is the inter-subunit helix-swapping of α12 and α13 between two non crystallographically related subunits responsible for the tetrameric structure. A similar helix swapping has been proposed to enable the formation of stable dimers in other proteins [16].

The mechanism of MtbICL function was elucidated based on the crystal structure of apo MtbICL and $MtbICL_{C191S}$in complex with glyoxylate and the non-reactive succinate analog 3-nitropropionate [15]. The conformation of apo MtbICL differs from its substrate analog bound forms, especially in regions that control access to the active site [1,15]. The first region is an active site loop (L_{185}ASEKKCGHLGG$_{196}$) that contains the ICL signature sequence (K_{189}K**C**GH$_{193}$) and the second region consists of the last 18 residues (P_{411}NSSTTALTGST**EE**GQFH$_{428}$) at the C-terminal end of the adjacent subunit (lid). The active site loop is flexible in the 'open' conformation where as in the substrate bound form it moves by 10–15 Å and attains a closed conformation. The closed conformation brings catalytic Cys191 of ICL signature sequence next to the substrate and completely closes off the active site from bulk solvent. The electrostatic interaction of Lys189 within the negative patches triggers movements in the active site loop to adopt the 'closed' conformation. Closure of the active site loop blocks the accessibility to the catalytic residues and invokes a movement of C-terminus lid (residues 411–428) of the adjacent subunit completing the catalytic conformation. It is not clear what invokes the movement of lid on top of the active site loop, locking it into a catalytic conformation? It appears that the two conserved glutamates in the lid region might play an important role during enzyme catalytsis [17]. Furthermore, apart from the residues of signature sequence (KKCGH), the function of ICL is also influenced by the histidine residue (His184) adjacent to the ICL signature sequence in *Escherichia coli*. The mutation of His184 to Lys, Arg, Leu or Gln resulted in either inactive ICL or a minimally active ICL [17].

Conformational analysis of MtbICL with respect to its stability and function is important and might help in the identification of suitable inhibitors. The biological phenomena depend on the molecular recognition and play a significant role in protein folding, stability and ligand binding. Understanding of the relationship between the structure of proteins, their stability, binding with micro or macromolecules etc. are useful to understand how biological systems work. This would facilitate drug discovery, such as in molecular mechanism and energetics of drug-macromolecules interactions or in order to estimate binding constants of two molecules [18,19].

In the present study, we systematically looked into the structural elements involved in the oligomerization and deactivation of MtbICL.

2. Experimental

2.1. Cloning and site directed mutagenesis

The cloning of full length MtbICL (MtbICL/$MtbICL_{\alpha12,\alpha13,\alpha14}$) has been described previously and was a kind gift from Dr. Ranjeet Kumar, Lucknow [20]. Truncated ICL without α14 ($tMtbICL_{\alpha12,\alpha13}$), $tMtbICL_{\alpha12}$ (without α13 and α14) and tMtbICL (without α12, α13 and α14) were amplified using suitable primers (Table 1). For all the samples, PCR reactions were carried out in a total volume of 50 μl with Platinum *Pfx* DNA polymerase (Invitrogen). The amplification condition was 94 °C-3 min; 94 °C-30 s, 53 °C-1 min, 68 °C-1 min (30 cycles); 68 °C-10 min. These amplified gene fragments were digested with *NheI* and *HindIII* and then ligated into the pET-23a (+) vector cut with the same enzymes. Competent *E. coli* DH5-α cells were transformed with the plasmid constructs and screened for positive clones. The mutant ICLs, $MtbICL_{H180A}$, $MtbICL_{Y89A}$, $MtbICL_{E423D}$, $MtbICL_{E424D}$, $MtbICL_{E423D\ E424D}$, $MtbICL_{E423A}$, $MtbICL_{E424A}$, $MtbICL_{E423AE424A}$, $MtbICL_{E423K}$, $MtbICL_{E424k}$, $MtbICL_{E423KE424K}$, $MtbICL_{E423L}$, $MtbICL_{E424L}$ and $MtbICL_{E423LE424L}$ were generated using mutagenic primer pairs (Table 1). The DNA sequencing of all the amplified genes confirmed the homogeneity of the sequences.

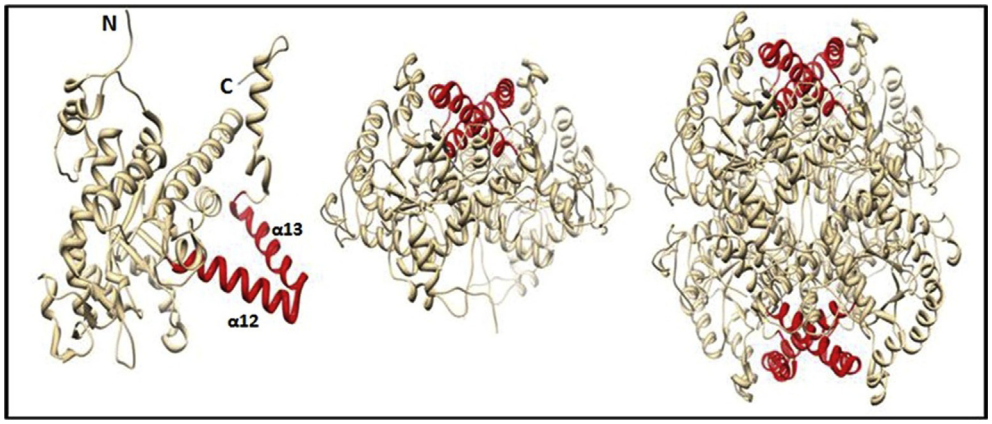

Fig. 1. Diagrammatic representation of the structure of MtbICL – Crystal structure of monomeric, dimeric and tetrameric MtbICL (left to right). The helices α12 and α13 involved in the oligomeization are marked as red. The structure was generated with the help of PISA server [28].