Mutational analysis of the *Mycobacterium tuberculosis* Rv1625c adenylyl cyclase: residues that confer nucleotide specificity contribute to dimerization

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Abstract The mycobacterial Rv1625c gene product is an adenylyl cyclase with sequence similarity to the mammalian enzymes. The catalytic domain of the enzyme forms a homodimer and residues specifying adenosine triphosphate (ATP) specificity lie at the dimer interface. Mutation of these residues to those present in guanylyl cyclases failed to convert the enzyme to a guanylyl cyclase, but dramatically reduced its adenylyl cyclase activity and altered its oligomeric state. Computational modeling revealed subtle differences in the dimer interface that could explain the biochemical data, suggesting that the structural and catalytic features of this homodimeric adenylyl cyclase are in contrast to those of the heterodimeric mammalian enzymes.

Key words: Adenylyl cyclase; Guanylyl cyclase; Rv1625c; Homology modeling; *Mycobacterium tuberculosis*

1. Introduction

The mammalian adenylyl cyclases that are regulated by the heterotrimeric G-proteins are large polypeptides containing 12 transmembrane spanning helices with two catalytic domains, C1 and C2. The catalytic domains of the mammalian enzymes have been characterized extensively both biochemically and structurally, and are active as heterodimers of the C1 and the C2 polypeptide regions, forming a single catalytic site at the interface [1]. This appears to be in contrast to the type III cyclases present in genomes from other organisms. The genome of *Mycobacterium tuberculosis* H37Rv, a parasite residing in host alveolar macrophages, contains 16 open reading frames that code for putative class III nucleotide cyclase genes [2,3]. Some of the mycobacterial genes bear a high degree of sequence similarity to mammalian adenylyl and guanylyl cyclases, and two of these genes, Rv1264 and Rv1625c, have been shown to have adenylyl cyclase activity [4–6] and function as homodimers.

The substrate selectivity of the type III nucleotide cyclases has been studied by mutational analysis and structure-based modeling, and residues that select for adenosine triphosphate (ATP) or guanosine triphosphate (GTP) have been identified [7]. Adenylyl cyclases have lysine (Lys-938 in type II C2) and aspartate (Asp-1018 in type II C2) residues in the catalytic cleft, which are replaced by glutamate and cysteine residues in guanylyl cyclases (Glu-925 and Cys-997 in retinal guanylyl cyclase). Switching of residues in a soluble [8] and membrane-bound guanylyl cyclase [9] to those in adenylyl cyclases led to the conversion of the guanylyl cyclases to adenylyl cyclases, but a similar exchange of residues in an adenylyl cyclase gave rise to a non-specific purine cyclase [8]. Substrate selectivity of cyclases from unicellular organisms has also been altered by similar mutations [10–12]. The active site of the mammalian enzymes lies at the dimer interface and the crystal structures of the inactive C2 homodimer and the C1-C2 heterodimer reveal a rotation of the C1 domain relative to C2 that optimizes substrate conversion [1]. However the role of substrate-specifying residues in the positioning of the two subunits, if at all, has not been studied.

The Rv1625c gene product comprises a protein with six transmembrane helices and a single cytosolic catalytic domain, which dimerizes to form a 12-transmembrane, homodimeric enzyme, with two catalytic centers, in contrast to the heterodimeric mammalian enzyme [6]. It therefore provides an opportunity to study biochemical and structural features that could be distinct from the mammalian enzymes. Based on the ability of the heterodimeric mammalian enzymes to convert their nucleotide specificity by mutational analysis, we decided to investigate the role that these residues present at the dimer interface play in Rv1625c to compare and contrast its properties with its mammalian counterparts. We show here, by biochemical analysis and computational modeling approaches, that residues that specify the substrate in mammalian nucleotide cyclases can alter the dimer-forming ability and interface of the Rv1625c cyclase. Our studies therefore suggest a new role for these residues in regulating the catalytic activity of homodimeric nucleotide cyclases, in their ability to subtly alter the dimeric interface of these enzymes.

2. Materials and methods

2.1. Bioinformatics

The Rv1625c sequence was aligned with representative members of the adenylyl and guanylyl cyclases and multiple sequence alignments were performed using Clustal X [13]. The amino acid sequences of the catalytic domains of several class III nucleotide cyclases were obtained from the Simple Modular Architecture Research Tool (SMART) database [14,15] (http://smart.embl-heidelberg.de/). The catalytic domain of class III nucleotide cyclases has a SMART accession number SM0044 (CycC).

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A three-dimensional model for the catalytic domain of Rv1625c was generated on the basis of the available crystal structures of rat type II C2 homodimeric adenylyl cyclase (Code: 1ab8), and C1 and C2 adenylyl cyclase subunits bound to Gsα (Code: 1azs). Simultaneous use of the three tertiary structures has been made in the modeling of Rv1625c using the COMPOSOR suite of programs [16–18] encoded in the SYBYL software (Tripos Inc., St. Louis, MO, USA). The dimeric model of Rv1625c has been generated on the basis of the dimeric crystal structures. The structural model has been energy minimized using the AMBER force-field [19]. SETOR program was used to view the structures and draw Fig. 4 [20].

2.2. Cloning, expression and purification of Rv1625c gene product

Primers (626f, 5′-AGGCGGCCATGGAGGCGGAGCAC-3′ and 1355r, 5′-GGCCCCGGGATAAAGCTTGGCGG-3′) were designed to the catalytic domain of Rv1625c, corresponding to amino acids 212–443 as per annotation at The Institute for Genomic Research (TIGR) website http://www.tigr.org, and based on multiple sequence alignment of several adenylyl and guanylyl cyclases. The product obtained from the polymerase chain reaction (PCR) using the cosmid MTCY0IB2 (Cole, S.T., Institute Pasteur, France) as template (10 ng), was cloned in the pCRII vector (Invitrogen) to generate the plasmid pCRII-Rv1625c$^{212-443}$ and the cloned insert sequenced. The insert was then cloned into pRSET-B vector (Invitrogen), using the *Nco*I–*Hin*dIII sites in the primers, to generate plasmid pRSET-Rv1625c$^{212-443}$, and the C43(DE3) derivative of *Escherichia coli* BL21(DE3) [21] was used for production of protein. The protein synthesized has an N-terminal hexa-histidine tag and was purified using Ni-NTA agarose (Qiagen). Purified protein was stored in 20 mM HEPES–NaOH buffer (pH 7.5) and 2 mM 2-mercaptoethanol (2-ME) containing 10% glycerol at −70°C until use. Enzyme remained active for months under these conditions. Protein estimation was performed by the method of Bradford with modifications [22]. An antibody was raised to the catalytic domain fused to glutathione S-transferase (data not shown), affinity purified against the catalytic domain protein and used for Western blot analysis.

2.3. Gel filtration analysis and cross-linking

Gel filtration was performed in 20 mM HEPES–NaOH buffer (pH 7.5), 5 mM 2-ME and 10% glycerol on an AKTA fast protein liquid chromatography (FPLC) system using a Superdex 200 column (25 cm×1 cm, Amersham Pharmacia Biotech) at a flow rate of 0.2 ml min^{-1}. The column was calibrated with bovine thyroglobin (669 kDa), IgG (150 kDa), serum albumin (66 kDa), ovalbumin (45 kDa) and cytochrome *c* (12.3 kDa).

Cross-linking was performed using 1 μg of purified protein in 50 mM HEPES–NaOH buffer, pH 7.5, 1 mM dithiothreitol (DTT) and 10% glycerol in the presence of 2 mM disuccinimidyl suberate (DSS) or an equivalent amount of the solvent, dimethyl sulfoxide. The reaction was allowed to proceed for 30 min at room temperature and was terminated by addition of 4× Laemmli loading dye. Samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by Western blotting using an affinity-purified antibody to the Rv1625c catalytic domain. Immunoreactive bands were visualized by enhanced chemiluminescence as described earlier [23].

2.4. Adenylyl and guanylyl cyclase assays

Adenylyl cyclase assay tubes were incubated at 25°C in a total volume of 50 μl, in 50 mM HEPES–NaOH buffer (pH 7.5), 1 mM DTT and 10% glycerol, for 10 min. Protein concentration used was 600 nM, and these conditions were found to allow linearity of product formation under the conditions of the assay. MnATP concentrations were varied up to a maximum of 2 mM and the free Mn concentrations in excess of the amount required for formation of metal-ATP, was kept at 10 mM. The concentrations of free metals and metal-ATP complexes in assays were calculated using WinmaxC (http://www.stanford.edu/~cpatton/maxc.html [24]). Guanylyl cyclase assays were carried out for 10 min at 37°C in the presence of 1 mM GTP and 10 mM $MnCl_2$, or 10 mM GTP and 50 mM $MnCl_2$. Assays were terminated by the addition of 50 mM sodium acetate buffer (pH 4.3) and boiling of the samples. Suitable volumes were used for cyclic nucleotide estimation with modifications [25]. The cAMP radioimmunoassay could detect 10 fmol of cAMP per tube. The cGMP assay was performed only after acetylation of samples and could detect 1 fmol of cGMP per tube. Enzyme kinetics data were fitted to the Michaelis–Menten equation or the Hill equation [26] using GraphPad Prism (San Diego, CA, USA). Where mentioned, values represent mean ± S.E.M. from experiments performed at least thrice.

2.5. Mutagenesis of Rv1625c

Plasmids pCRII-Rv1625c$^{212-443}$ or pRSET-Rv1625c$^{212-443}$ were used for site-directed mutagenesis of the catalytic domain. The first primer mutated a lysine (Lys-296) to an aspartate residue and had the sequence 5′-TGGAGAAAATCGAGGTCAGCGGGGA-3′ (primer KE). The second primer, DC, had the sequence 5′-TCGCCCCACACGCAGTAGAAGAACCG-3′ and mutated an aspartate (Asp-365) to a cysteine residue. The two products generated using primer KE and primer 1355r, and primer DC and primer 626f, were taken for overlap PCR at 68°C for 10 cycles. An aliquot of this PCR was re-amplified with primers 626f and 1355r and the product was cloned in to pCRII vector (Invitrogen) to generate the plasmid pCRII-Rv1625c-K→E. Sequencing showed the presence of only the K296E mutation, though both mutations were expected. The D365C mutant was obtained by a modification of the *Dpn*I digestion method of mutagenesis [27]. pCRII-Rv1625c-K→E was used as a template and sequencing confirmed the presence of the additional D365C mutation and this plasmid was called pCRII-Rv1625c-KD→EC. Both these mutants were subcloned into the pRSET-B vector at the *Nco*I–*Hin*dIII sites (Invitrogen) to generate pRSET-KE and pRSET-KDEC. The D365C single mutant was obtained by ligating an *Nco*I–*Sac*II fragment from pCRII-Rv1625c$^{212-443}$ and a *Sac*II–*Hin*dIII fragment from pCRII-Rv1625c-KD→EC to generate pRSET-DC. The F363R mutation was generated by similar *Dpn*I-based mutagenesis on the pRSET-KDEC plasmid using the FR primer 5′-TCGACGGTTCCGCTACTGCGTGTGGG-3′ to generate plasmid pRSET-KFD→ERC. The mutant proteins are referred to as K→E, D→C, KD→EC and KFD→ERC throughout the text. All mutants have been sequenced to rule out the presence of other missense mutations. Proteins were expressed in the *E. coli* C43(DE3) strain and purified using procedures adopted for the wild-type protein. Cross-linking and gel filtration analysis were performed as described above for the wild-type protein.

3. Results and discussion

3.1. Expression and purification of the catalytic domain of Rv1625c

The catalytic domain of Rv1625c has been shown to aggregate at high protein concentrations, thereby reducing its catalytic activity [6]. We therefore expressed a region of the protein that is more closely defined at its N-terminus by residues that show similarity to other adenylyl and guanylyl cyclases (Fig. 1). Also shown in Fig. 1 are residues that confer nucleotide specificity in adenylyl and guanylyl cyclases, and as can be seen, the catalytic domain of Rv1625c shows similarity to both adenylyl and guanylyl cyclases in regions around the substrate-specifying residues identified by computational analysis of cyclase sequences [28].

The expressed protein was purified (Fig. 2A) and adenylyl cyclase assays were performed in the presence of Mn as metal cofactor. The activity showed a sigmoidal substrate response curve (Fig. 2B), indicating that the two catalytic centers in the enzyme interact with each other, in contrast to the classical hyperbolic catalysis seen with the purified C1 and C2 domains of the mammalian adenylyl cyclase, which form a single catalytic center [29]. The Hill coefficient calculated from the data was 3.9 ± 0.6 and the apparent K'_{MnATP} was 363 ± 43 μM, indicating a highly cooperative interaction between the two substrate binding regions in the protein.

The expressed protein was subjected to gel filtration analysis and, as shown in Fig. 2C, eluted largely as a monomeric species with some amount of dimer. Cross-linking of the protein with DSS was in agreement with these observations (Fig. 2C). Cross-linking was also observed in the presence of

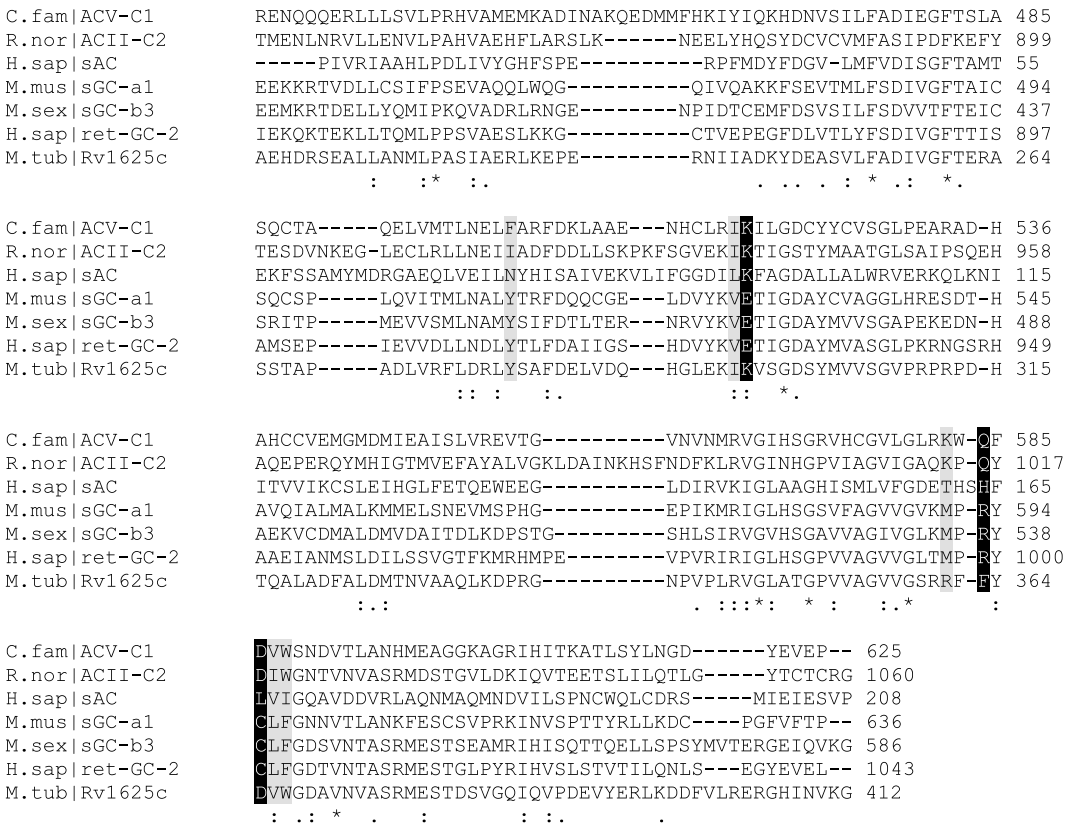

Fig. 1. Sequence analysis of the Rv1625c catalytic domain. Class III nucleotide cyclase catalytic domain amino acid sequences were obtained from the SMART database and multiply aligned using Clustal X. Residues specifying the nucleotide demonstrated biochemically are highlighted in black, and those additionally identified through sequence analysis are highlighted in gray [28]. C. fam: *Canis familiaris*; R. nor: *Rattus norvegicus*; H. sap: *Homo sapiens*; M. mus: *Mus musculus*; M. sex: *Manduca sexta*; M. tub: *M. tuberculosis*. The proteins used for the alignment are as follows ACV-C1:P30803; ACII-C2:P26769; sAC:Q96PN6; sGC-a1:Q9ERL9; sGC-b3:O76340; ret-GC-2:P51841; and Rv1625c:O30820. Notice the similarity of the Rv1625c to both adenylyl and guanylyl cyclases.

MnATP (data not shown), but no cross-linked product was observed with disuccinimidyl tartarate, indicating that cross-linking was specific and depended on the correct positioning of lysine residues in the protein (data not shown). The fact that this protein was less prone to aggregation at high concentrations, in contrast to an earlier described catalytic domain construct [6], allowed us to study residues that confer substrate selectivity and are required for maintaining the oligomeric status of the enzyme as described below.

3.2. *Residues that confer nucleotide selectivity also are involved in productive dimer formation*

The crystal structure of the C1-C2 domains of the mammalian enzyme revealed that a main chain carbonyl interacts with the adenine N6 and helps in contributing to the specificity of nucleotide binding, along with the substrate-specifying residues, lysine and aspartate [30]. Therefore, mutation of the lysine and aspartate residues in adenylyl cyclases to a glutamate and a cysteine present in guanylyl cyclases converts these enzymes to a non-specific cyclase [8], but increases the K_m for ATP dramatically. Given the significant sequence similarity of Rv1625c with both adenylyl and guanylyl cyclases, we wished to evaluate the importance of the substrate-specifying residues (Lys-296 and Asp-365) in Rv1625c, by mutational analysis, in an attempt to convert it to a guanylyl cyclase.

Wild-type Rv1625c showed no guanylyl cyclase activity using as much as 10 mM GTP as substrate [6] (data not shown). We generated four mutant proteins, K296E and D365C with single mutations in residues that interact with either ATP or GTP in adenylyl and guanylyl cyclases respectively, and K296E/D365C with both mutations (Fig. 1). In addition, a third mutation shown to be required for efficient conversion of an adenylyl cyclase to a guanylyl cyclase [7,8] was also generated and involved the mutation of a phenylalanine residue to an arginine residue (K296E/F363R/D365C triple mutant). All mutant proteins were purified and tested for catalytic activity. Interestingly, none of the mutant proteins demonstrated any guanylyl cyclase activity, though the assay using radioiodinated cGMP that we use is capable of detecting fmol concentrations of cGMP per tube [23]. Even concentrations of GTP as high as 10 mM failed to show any formation of cGMP (data not shown).

However, all proteins demonstrated some adenylyl cyclase activity, but much lower than that of the wild-type enzyme. Moreover, mutant proteins displayed classical Michaelis–Menten kinetics, as opposed to the sigmoidal nature of the wild-type enzyme (Fig. 3A), indicating that the communication between the two catalytic sites was lost, perhaps as a consequence of altered positioning of the two subunits in the region of the catalytic center. The affinity of the enzymes for MnATP did not differ dramatically, but the V_{max}, and, as a consequence, the specific activities of the mutant proteins, was markedly compromised, as compared to the wild-type enzyme.

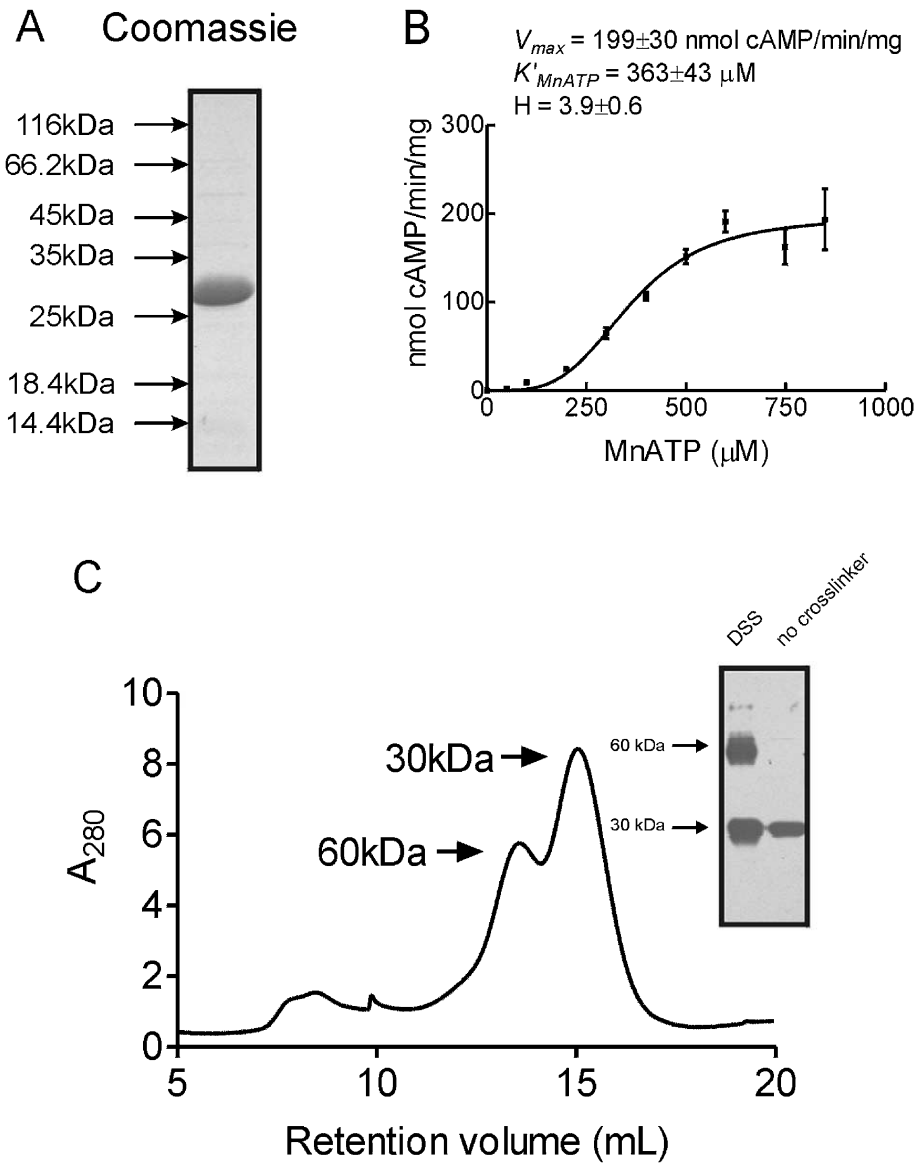

Fig. 2. Catalytic activity and dimeric nature of the catalytic domain of Rv1625c. A: The catalytic domain (5 μg) of Rv1625c was purified subjected to SDS–gel electrophoresis and stained with Coomassie. B: Adenylyl cyclase assays were performed in the presence of a fixed concentration of 10 mM free Mn and varying concentrations of MnATP as indicated. Values shown are representatives of assays performed at least thrice. C: Gel filtration analysis of the purified Rv1625c catalytic domain protein was performed on a Superdex 200 column. Protein eluting at positions corresponding to a monomer and a dimer are shown. Inset: Cross-linking of the purified catalytic domain of Rv1625c was performed with DSS and subjected to SDS–PAGE and Western blot analysis with an affinity-purified antibody to the catalytic domain of Rv1625c.

The low adenylyl cyclase activity of the mutant proteins was not because of general misfolding as seen from their gel filtration profiles (see below) where they eluted with well-defined hydrodynamic parameters. In addition, we did not observe any change in the expression levels or the solubility of the wild-type and mutants proteins, again indicating that mutations had not compromised the folding in *E. coli* cells. We therefore decided to check if the loss of activity was due to inability of the mutants to form functional dimers.

Cross-linking and gel filtration analysis were performed with the wild-type and mutant proteins (Fig. 3B). Cross-linking experiments (Fig. 3B, insets) showed that KD→EC and D→C mutants could cross-link to form dimers in the presence of DSS, indicating that K296 may not be directly involved in forming cross-links. No dimers were seen with the K→E mutant under similar conditions, but overexposure of the Western blot did show a small amount of dimer (data not shown). Gel filtration analysis demonstrated a difference in the oligomeric states of the mutant proteins (Fig. 3) in agreement with the cross-linking results. The gel filtration profile of the K→E mutant was similar to that of the wild-type enzyme (Fig. 3), showing the presence of a significant amount of monomeric protein species. In contrast, the KD→EC double mutant and D→C single mutant contain large amounts of the dimer, with very little monomeric species. This dimerization was not due to the formation of a disulfide bridge between the two single cysteine residues present in the mutant proteins, since purified proteins were always stored in reducing agent (see Section 2). The triple mutant, KFD→ERC was found to exist predominantly as a monomer and showed very little

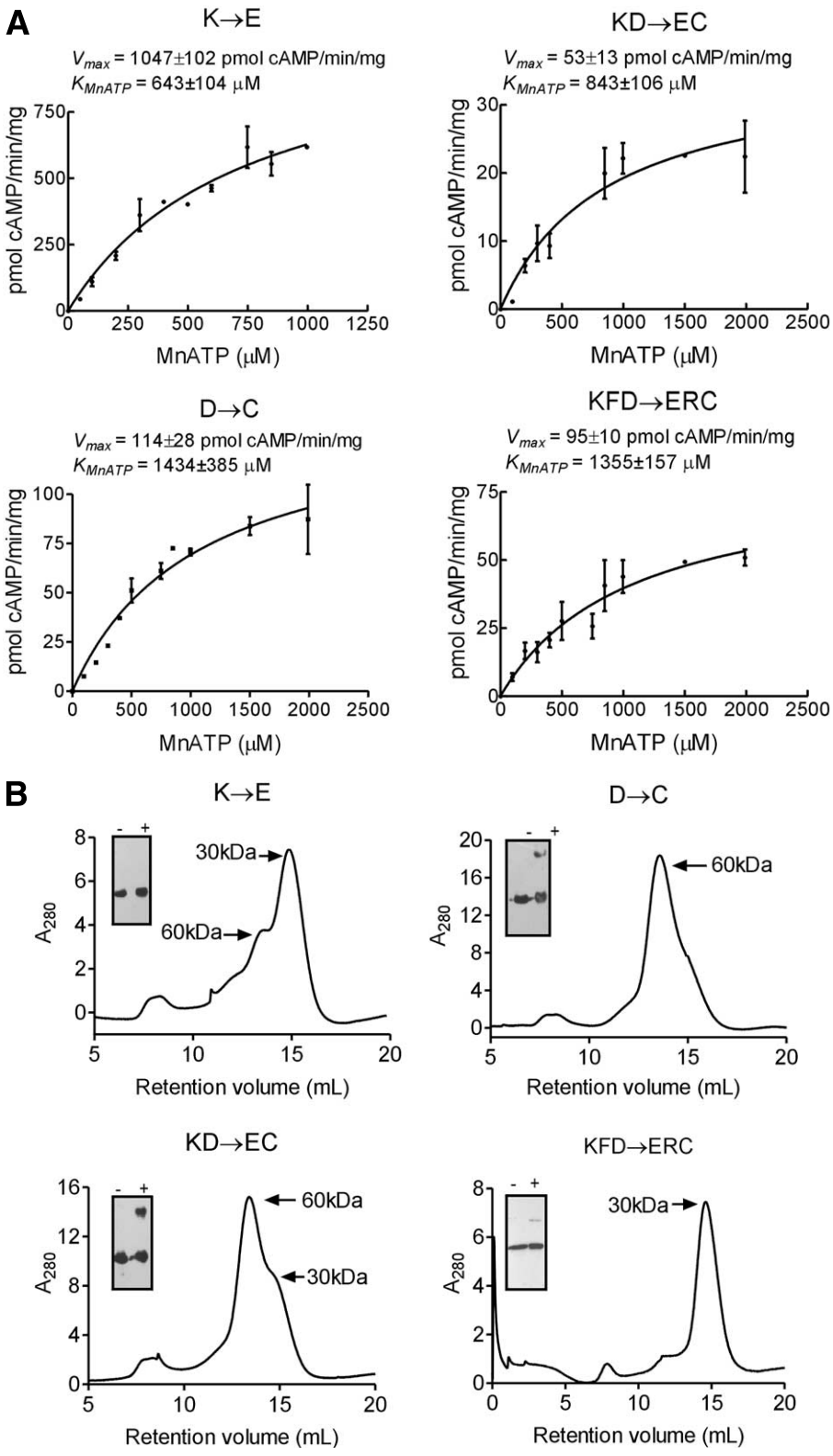

Fig. 3. Catalytic activity and gel filtration analysis of mutant Rv1625c proteins. A: Purified proteins (2 μg) were used for adenylyl cyclase assays using varying concentrations of MnATP as substrate in the presence of a fixed concentration of 10 mM Mn. Data shown are representatives of assays performed thrice. B: Purified proteins (75–100 μg) were subjected to gel filtration analysis. The K→E and KFD→ERC mutant proteins existed predominantly as monomers, while the D→C and KD→EC mutant proteins had more of the dimeric species. Proteins corresponding to monomer (30 kDa) and dimer (60 kDa) are seen. Insets: Purified mutant proteins (1 μg each) were treated with dimethyl sulfoxide (lanes marked −) or cross-linked with DSS (lanes marked +) and subjected to Western blot analysis using an affinity-purified antibody to Rv1625c.

dimer formation on cross-linking. These results therefore suggest that mutation of the substrate-specifying residues, present at the dimer interface of Rv1625c, also regulate the extent of dimer formation, and suggest an additional role for these residues in forming a productive catalytic cleft between the two monomers of the enzyme, that can allow correct binding of ATP and allow conversion to cAMP.

3.3. Computational modeling of the Rv1625c catalytic domain

In order to put these observations in a structural context, we modeled the homodimeric catalytic domain of Rv1625c, using information from available crystal structures [30,31]. As the sequence identity between the cyclase domains of Rv1625c and known structures of adenylyl cyclases was sufficiently high (32–36%; [16]), most of the regions could be modeled reliably. Fig. 4A shows the overall fold of the dimeric model of Rv1625c. Structural regions with contiguous non-conservative substitutions in amino acid sequence compared to cyclases of known structure are highlighted in red. Most of the regions that show maximum deviation are solvent-exposed loops and terminus regions of helices and β-strands. The last 30 residues of Rv1625c show no high similarity with the corresponding regions of cyclases of known structure, and this region is hypervariable even amongst the cyclases of known structure.

The critical residues responsible for providing substrate selectivity, Lys-296 and Asp-365, were modeled in the conformations similar to that seen in the crystal structures (Fig. 4B). Hence, they appear to be involved in an intra-chain salt bridge formation, and are involved in stabilization of the tertiary structure of each subunit. When the lysine or aspartate residues in Rv1625c are mutated to equivalent residues in guanylyl cyclases (glutamate or cysteine), favorable intra-subunit interactions between these two residues would be lost, possibly leading to instability of the tertiary structure of the individual polypeptide chains, thereby reducing catalytic activity and altering dimer formation and positioning of the two subunits as we have observed experimentally.

While many of the putative interfacial residues in Rv1625c are conserved with respect to the crystal structures of the mammalian enzymes, there are some differences. Most significantly, phenylalanine at position 363 is present in the interface region. The equivalent residue in most of the adenylyl cyclases is glutamine and that in guanylyl cyclases is arginine (Fig. 1). Thus, the loss of Phe-363 in the KFD→ERC mutant protein could abolish the stacking interaction between the aromatic rings of Phe-363 from the two subunits. Moreover, the introduction of the charged residue arginine could further disable homodimer formation, as is evident from the predominantly monomeric nature of the triple mutant protein.

In spite of the higher overall sequence similarity to guanylyl cyclases, GTP could not be used as a substrate by Rv1625c, after replacement of ATP-specifying residues to those at equivalent positions in guanylyl cyclases. The fact that adenylyl cyclases with a single catalytic site showed some guanylyl cyclase activity by similar mutations, indicates that substrate-specifying residues in Rv1625c play an additional role in its quaternary structure, distinct from that in mammalian enzymes. Substrate specificity change in the homodimeric retinal receptor guanylyl cyclase could take place with only two mutations [9], while the heterodimeric soluble guanylyl cyclase required three mutations [8]. Interestingly, mutation of this third residue (Phe-363) to arginine in Rv1625c, which stabilizes the glutamate residue in mammalian guanylyl cyclases, was found to be detrimental to dimerization as seen in gel filtration experiments and as predicted by homology modeling (Figs. 3 and 4). This again suggests subtle structural differences between the single catalytic centered mammalian adenylyl and soluble guanylyl cyclases and the double catalytic centered receptor guanylyl cyclases and Rv1625c, and points to the uniqueness of the Rv1625c substrate binding pocket.

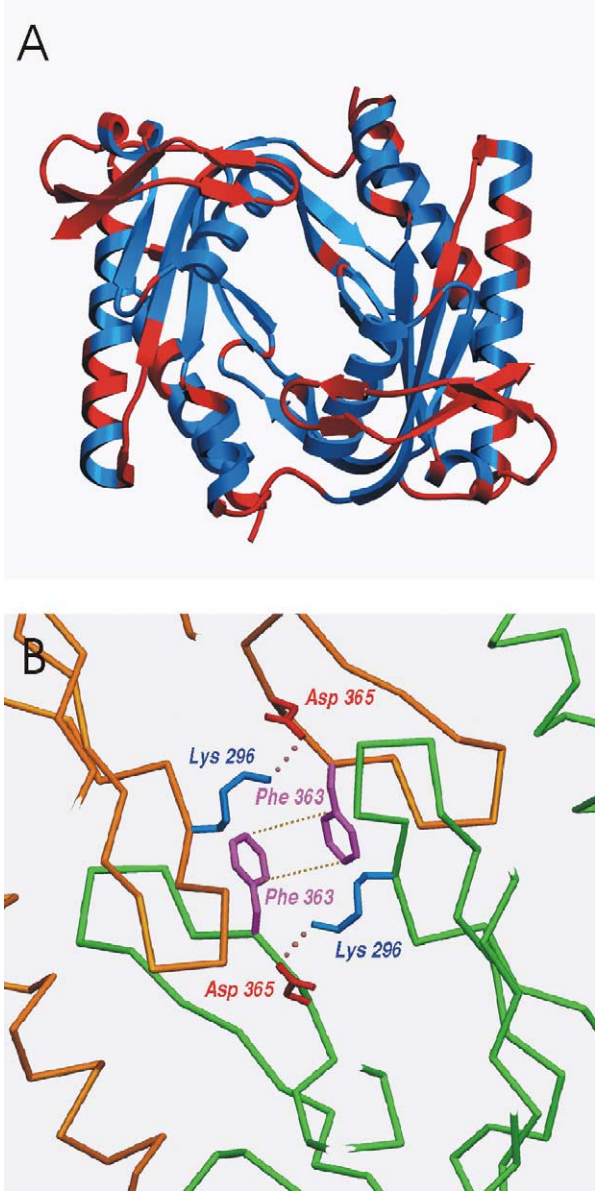

Fig. 4. Three-dimensional model of dimeric Rv1625c. A: Ribbon representation of the model of Rv1625c generated on the basis of the known related structures. The structural regions with deviation, in terms of local sequence similarity with the cyclases of known structure, are shown in red. B: Close-up of the inter-protomer interface of the three-dimensional model of dimeric Rv1625c. The C-alpha traces of the two subunits are shown in brown and green respectively. Phe-363 and the catalytic residues Lys-296 and Asp-365 from the two subunits are shown. The predicted salt bridge between these residues and an interaction between the aromatic groups of the side chains of Phe-363 residues from the two subunits are represented by dotted spheres. The figure was generated using SETOR [20].

We did not expect residues involved in ATP binding to regulate the oligomeric status of the protein despite the fact that they lie at the dimer interface. The high cooperativity observed with MnATP in the wild-type enzyme, however, indicates that the protein undergoes a significant conformational change upon binding the first molecule of MnATP. The single mutation of K→E abolished the cooperativity of the enzyme with respect to ATP binding, and also reduced catalytic activity by almost 200-fold. More dramatic reductions in catalytic activity were seen with the additional mutant proteins. Given the somewhat smaller change in the apparent K_m for ATP, it is likely that the accurate juxtaposing of catalytic residues brought about after binding of substrate is lacking in the mutants, that leads to a reduced activity, rather than a reduced binding of substrate. This clearly implies a greater role for substrate-specifying residues in Rv1625c than has been observed in the mammalian enzymes.

The presence of excess GTP in the reaction did not inhibit the adenylyl cyclase activities of either the wild-type or the mutant proteins, nor was guanylyl cyclase activity seen even with high (10 mM) concentrations of GTP. Therefore, Rv1625c and the mutant proteins are unable to bind GTP, and utilize it as a substrate. Additional residues suggested to provide substrate specificity have been identified, and mutations of these residues (indicated in Fig. 1; [28]) could perhaps alter substrate specificity in Rv1625c. Indeed, the requirement of K and D for optimal positioning of catalytic residues in Rv1625c raises the question as to whether this enzyme can be converted to a guanylyl cyclase without altering the K and E residues, and by mutating other residues, as described by Hannenhalli and Russell [28]. The catalytic site of nucleotide cyclases is formed at the dimer interface and therefore it is attractive to suggest that additional residues required for catalysis may also regulate dimer formation and structure of Rv1625c. These could include residues that interact with the metal ions essential for catalysis, and given the apparent difference in the dimer interface of Rv1625c, it is possible that regulation of this enzyme by metals may differ from the mammalian enzymes as well. Such studies are currently ongoing in the laboratory.

Rv1625c is one of the many putative genes in the genome of *M. tuberculosis* H37Rv that has a high sequence similarity to the class III nucleotide cyclases. As we have shown here, despite the high (~60% similarity to guanylyl cyclases) sequence similarity to the mammalian enzymes, Rv1625c has unique properties that have not been reported in any other class III cyclase so far. These differences could in principle be exploited to understand in greater detail the evolution and structural features of the large family of nucleotide cyclases.

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