ABSTRACT

Pantothenate kinase (PanK) is a ubiquitous and essential enzyme that catalyses the first step in the universal Coenzyme A (CoA) biosynthetic pathway. In this step pantothenate is converted to phosphopantothentate, which subsequently forms CoA in four enzymatic steps. Three types of PanKs have been identified in bacteria, with variations in distribution, mechanism of regulation, cofactor requirement and affinity for substrates. As part of a major programme on mycobacterial proteins in our laboratory, studies on type I PanK from *Mycobacterium tuberculosis* (*Mt*PanK) have been carried out previously. This investigation involved, apart from biochemical studies, structure determination of twenty-one independent crystals of binary and ternary complexes of *Mt*PanK involving CoA, the ATP analogue AMPPCP, the GTP analogue GMPPCP, ADP, GDP, pantothenate, phosphopantothenate, citrate, pantothenol and nonyl pantothenamide. Analysis of these structures brought out the robustness of the mycobacterial PanK when compared to its *E. coli* homologue. It was observed that while the protein structure remained relatively rigid in all the *Mt*PanK structures, the ligands exhibited substantial movement in the pre-formed pocket during the course of catalysis. This observation was unlike that seen in *Ec*PanK, where the protein molecule underwent conformational changes during enzyme action. Also, the feedback inhibitor, CoA, showed a higher binding affinity to *Mt*PanK compared to that to *Ec*PanK. The differences exhibited by these homologous proteins despite sharing 52% sequence identity were surprising and merited further study. To this end, mutants of *Mt*PanK were prepared, their structures solved, and solution studies related to binding and activity of these mutants were carried out. Apart from this, supplemental molecular dynamics (MD) studies were carried out on *Mt*PanK and *Ec*PanK and the mutants of *Mt*PanK.

Structural studies were carried out using conventional tools and techniques of macromolecular crystallography. The microbatch under-oil method was used for crystallisation

in all cases. Data were collected at a home-source on a MAR 345 image plate mounted on a Bruker MICROSTAR ULTRA II Cu Kα rotating-anode X-ray generator or using a CCD detector (MARMosaic 225) on the synchrotron X-ray beamline BM14 at the European Synchrotron Radiation Facility, Grenoble, France. Data were processed using MOSFLM and SCALA and the structures were solved by the molecular replacement method using PHASER from the CCP4 suite. Refinement was carried out using REFMAC and manual model building was performed employing COOT. Structures were validated using PROCHECK. Thermal shift assay was used to study binding of CoA to the mutants. A radioactive assay and an enzyme coupled assay were employed to measure the activity of the mutants.

To begin with, the high affinity of CoA to *Mt*PanK was sought to be disturbed by disrupting the binding site using mutations. Therefore, two conserved phenylalanine residues of the hydrophobic binding site were targeted and two point mutants and a double mutant were constructed. Solution studies on the three mutants confirmed the reduction in CoA binding affinity and also that of activity to some extent. Structure solution of the mutants showed that apart from local rearrangements, the mutations led to partial or complete transition of the structure to that seen in *Ec*PanK. Concerted movement was observed in the dimerisation region and the nucleotide binding region.

To further understand this transformation and as a complementary effort to the studies on the CoA binding region mutants, mutations were made in the substrate binding regions of *Mt*PanK such that non-conservatively substituted residues were replaced with those found in *Ec*PanK. Solution studies on these mutants showed that CoA binding affinity was minimally affected by the mutations, while activity was reduced to some extent. In all, six structures were solved, half of which were CoA-free and showed partial or complete transformation to an Ec-like conformational state. Concerted movement was seen in another loop along with that seen in the dimerisation interface and the nucleotide binding region. The structures brought to light

the changes in conformations of certain residues and their interactions that makes the Ec-like state feasible in *Mt*PanK. Put together, these studies showed how small perturbations like those caused by point mutations could bring about global transformations in the structure of *Mt*PanK. They also suggest that *Mt*PanK may be able to utilise the nucleotide binding pocket as seen in *Ec*PanK in the transformed structures. This is an aspect that may be important in relation to drug designing.

The results obtained from the mutational studies were supplemented by MD simulations on *Mt*PanK, *Ec*PanK and mutants of *Mt*PanK. These studies helped delineate an invariant core common to *Mt*PanK, *Ec*PanK and the *Mt*PanK mutants. They also showed that wild-type *Ec*PanK is indeed more flexible than wild-type *Mt*PanK. Furthermore, MD simulations showed the impact of sequence on the structure of the *Mt* enzyme. Minor sequence changes appeared to influence different structural elements, including those far away from the sites of mutation. Thus, it would seem that an ensemble of structures is accessible to the PanK molecule and the selection of an appropriate conformation is based on the requirement brought about by mutations or ligand binding.

Apart from the studies on *Mt*PanK, structural studies on argininosuccinate lyase from *Mycobacterium tuberculosis* were also carried out. The native structure along with that bound to the substrate and products helped propose a catalytic mechanism based on previously available information and present studies. This investigation is presented in an appendix.