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Structure and Reaction Mechanism of L-Rhamnulose Kinase from *Escherichia coli*

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Bacterial L-rhamnulose kinase participates in the degradation of L-rhamnose, which is ubiquitous and particularly abundant in some plants. The enzyme catalyzes the transfer of the γ-phosphate group from ATP to the 1-hydroxyl group of L-rhamnulose. We determined the crystal structures of the substrate-free kinase and of a complex between the enzyme, ADP and L-fructose, which besides rhamnulose is also processed. According to its chainfold, the kinase belongs to the hexokinase-hsp70-actin superfamily. The closest structurally known homologue is glycerol kinase. The reported structures reveal a large conformational change on substrate binding as well as the key residues involved in catalysis. The substrates ADP and β-L-fructose are in an ideal position to define a direct in-line phosphoryl transfer through a bipyramidal pentavalent intermediate. The enzyme contains one disulfide bridge at a position where two homologous glycerol kinases are regulated by phosphorylation and effector binding, respectively, and it has two more pairs of cysteine residues near the surface that are poised for bridging. However, identical catalytic rates were observed for the enzyme in reducing and oxidizing environments, suggesting that regulation by disulfide formation is unlikely.

Keywords: hexokinase-hsp70-actin superfamily; induced fit; in-line phosphoryl transfer; rhamnose degradation; X-ray diffraction

Introduction

The sugar L-rhamnose (6-deoxy-L-mannose) is a common natural deoxy-hexose, which is ubiquitous and rather abundant in some plants.[1] *Escherichia coli* processes the sugar using the enzymes of the L-rhamnose regulon, which are L-rhamnose permease, L-rhamnose isomerase, L-rhamnulose kinase, L-rhamnulose-1-phosphate aldolase and two regulatory proteins.[2] Here, we are concerned with L-rhamnulose 1-kinase (RhuK, EC 2.7.1.5), which is encoded by the *rha*B gene and catalyzes the phosphorylation of L-rhamnulose, as depicted in Figure 1. The product L-rhamnulose-1-phosphate is subsequently split by the aldolase into L-lactaldehyde and dihydroxyacetone phosphate.

The amino acid sequence of RhuK shows 25% identity with fuculose kinase,[2] which has been suggested to be a member of the hexokinase-hsp70-actin superfamily.[3] In that case, RhuK should also belong to this group. All superfamily members share a common characteristic five-stranded β-sheet occurring in the N-terminal as well as in the C-terminal domains.[4–14] Moreover, they have a common ATP-binding site in a deep cleft between the two domains, from where the γ-phosphoryl group is transferred either to a water molecule or to a substrate molecule located deeper in the same cleft. Given the common chainfold repeat and ATP site, the structural similarity within the superfamily is significant, indicating that the members have a common evolutionary origin. A large induced fit accompanying the reaction was proposed first for hexokinase,[15] and later for others. The adenosyl moiety of ATP binds to the C-terminal domain, whereas the phosphate groups are bound to both domains. Interestingly, the phosphates bind to the short connections between the first two strands of the characteristic β-sheets of each domain. This ATP-binding motif

Abbreviations used: apo RhuK, enzyme without a bound ligand; holo RhuK, enzyme with bound ADP and β-L-fructose; RhuK, L-rhamnulose 1-kinase: here the enzyme from *E. coli* BL21(DE3); SeMet, L-selenomethionine.

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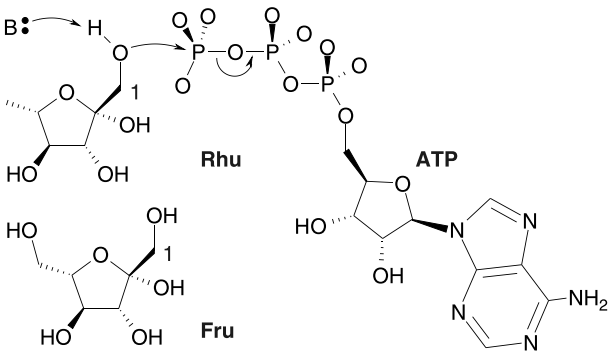

Figure 1. Reaction scheme showing the structures of the two substrates β-L-rhamnulose and β-L-fructose. The suggested in-line phosporyl transfer is indicated.

differs significantly from the P-loop occurring in numerous other kinases.[16]

Descending from the same ancestor, it seems likely that at least the kinases among the superfamily members follow a common reaction mechanism. On the basis of an observed configurational inversion in glycerol kinase and acetate kinase, a direct in-line phosphoryl transfer mechanism has been proposed.[17] Some time ago, an alternative triple displacement mechanism running through two covalently linked enzyme phosphate intermediates was discussed for acetate kinase.[18,19] This proposal was abandoned, however, after a recently established enzyme structure revealed a putative acetate molecule poised for an in-line transfer.[20] Following the structures of L-rhamnose isomerase[21] and L-rhamnulose-1-phosphate aldolase,[22] which act before and after RhuK in the L-rhamnose degradation pathway, we report the structure of RhuK in an open as well as in a closed conformation, compare it with the related kinases and discuss the mechanism of phosphorylation.

Results and Discussion

Structure determination

The *rha*B gene was isolated from chromosomal DNA of *E. coli* BL21(DE3) cells and overexpressed in *E. coli* JM105. A sequence comparison with the *rha*B gene of *E. coli* strain K12[23] showed 17 silent point mutations and the six exchanges Q310R, S320A, E343D, T344E, T356M and Q477L, all of which were found to be in insignificant locations on the surface. RhuK consists of 489 amino acid residues with a mass of 54,110 Da. The enzyme is monomeric according to its apparent mass of 52 kDa in size-exclusion chromatography (data not shown). Since none of the crystallization attempts with wild-type RhuK was successful, we produced two triple mutants by exchanging charged side-chains by alanine[24] at predicted surface loops.[25,26] Of these, the variant E69A-E70A-R73A yielded suitable crystals and retained full catalytic activity. The holo (ligated) and the apo (unligated) RhuK crystals grew under identical conditions but belonged to different space groups, namely $P2_12_12_1$ and $P2_1$, respectively. RhuK was labeled with L-seleno-methionine (SeMet) using the pathway inhibition method.[27] Electrospray ionization mass spectroscopy (ESI-MS) showed a mass difference between labeled and unlabeled RhuK that corresponded to 76% SeMet incorporation. The structure was established for a SeMet-labeled complex between RhuK, ADP and β-L-fructose, which in the following is called holo RhuK.

For phasing, multiwavelength anomalous X-ray diffraction data (MAD) were collected for a SeMet-labeled holo RhuK crystal (Table 1). The data yielded eight selenium sites out of the expected ten. These sites were used to produce an initial electron density map at 2.2 Å resolution, which revealed the whole model except for Met1, loop 223–226 and the C-terminal residues 481–489. A model containing residues 2–480 was then built and refined to 1.88 Å resolution, yielding good-quality indices (Table 2) and somewhat elevated *B*-factors around position 223. A ribbon representation of the enzyme is depicted in Figure 2. A further data set was collected with unlabeled RhuK with bound ADP and β-L-fructose, which assumed the same crystal form but did not quite reach the resolution of the SeMet-labeled data (Table 1). The unlabeled model was refined, resulting in essentially the same structure as the SeMet-labeled holo RhuK (Table 2). We used these data only to confirm the SeMet-labeled structure and do not refer to them explicitly in the following analysis.

Subsequently, the structure of apo RhuK was established by molecular replacement. However, an initial trial with the full holo RhuK as a search model failed. Therefore, the procedure was carried out in two steps: first with the N-terminal domain and then with the C-terminal domain using the best solution for the N-domain as a fixed structure part. The result showed that apo RhuK assumes an open conformation, differing appreciably from the closed conformation of the holo enzyme.

The two crystal forms of holo and apo RhuK are closely related, as they show essentially the same packing scheme. While the $P2_12_12_1$ crystals have one holo RhuK molecule in the asymmetric unit and a solvent content of only 35% (v/v), the $P2_1$ crystals contain two asymmetric apo RhuK molecules and have a solvent content of 40% (v/v). The unit cells of the two crystal forms are very similar. The three mutations required for crystal formation turned out to be on a protruding loop far away from the active center. They participate in three packing contacts of the $P2_12_12_1$ crystals, and in three contacts of the $P2_1$ crystals, explaining their important role in crystal formation.