The basis for non-canonical ROK family function in the \(N\)-acetylmannosamine kinase from the pathogen \textit{Staphylococcus aureus}

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Running title: \textit{\(N\)-Acetylmannosamine kinase from \textit{Staphylococcus aureus}}

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\textbf{ABSTRACT}

In environments where glucose is limited, some pathogenic bacteria metabolise host derived sialic acid as a nutrient source. \(N\)-Acetylmannosamine kinase (NanK) is the second enzyme of the bacterial sialic acid import and degradation pathway and adds phosphate to \(N\)-acetylmannosamine using ATP to prime the molecule for future pathway reactions. Sequence alignments reveal that Gram-positive NanK enzymes belong to the Repressor, Open-reading frame, Kinase (ROK) family, but many lack the canonical Zn-binding motif expected for this function, and the sugar-binding ExGH motif is altered to ExGY. As a result, it is unclear how they perform this important reaction. Here, we study the \textit{Staphylococcus aureus} NanK (SaNanK), which is the first characterisation of a Gram-positive NanK. We report the kinetic activity of SaNanK along with the ligand-free, \(N\)-acetylmannosamine-bound and substrate analog \(N\)-acytlyglucosamine-bound crystal structures (2.33, 2.20 and 2.20 Å resolution, respectively). These demonstrate, in combination with small-angle X-ray scattering, that SaNanK is a dimer that adopts a closed conformation upon substrate binding. Analysis of the ExGY motif reveals that the tyrosine binds to the \(N\)-acetyl group to select for the ‘boat’ conformation of \(N\)-acetylmannosamine. Moreover, SaNanK has a
stacked arginine pair coordinated by negative residues critical for thermal stability and catalysis. These combined elements serve to constrain the active site and orient the substrate in lieu of Zn binding, representing a significant departure from canonical NanK binding. This characterisation provides insight into differences in the ROK family and highlights a novel area for antimicrobial discovery to fight Gram-positive and S. aureus infections.

Pathogenic bacteria import and utilise a wide variety of carbohydrates. In environments where glucose is limited, amino sugars such as sialic acids are important nutrient sources. In general, the bacterial sialic acid catabolic pathway is a validated antimicrobial target (1-4), since sialic acids are scavenged from mucins that coat the gastrointestinal and respiratory tracts of humans (5) and are imported (6-8) and used by pathogenic bacteria as a carbon, nitrogen and energy source when they colonise these niches (1,9-11). This is particularly the case in mucosal surfaces, where the amino sugar N-acetylmannosamine and the sialic acid, N-acetylneuraminic acid, are widely incorporated into glycoconjugate mucins and cell surfaces [1]. These carbohydrates can be scavenged from host glycoconjugates and metabolised by mucosal pathogens, such as Staphylococcus aureus [2], as a nutrient source [3-6].

Just as the phosphorylation of glucose in the early phase of glycolysis (sometimes referred to as the “energy investment phase”) drives otherwise unfavourable reactions in the payoff phase as glucose is catabolised, in the bacterial sialic acid import and degradation pathway (Fig. 1A), phosphorylation serves to prime the amino sugar for downstream catabolism through glycolysis, or anabolism by synthetic pathways, including cell wall synthesis (10,12). Here, we focus on the second step of the catabolic pathway: the phosphorylation of N-acetylmannosamine catalysed by N-acetylmannosamine kinase (henceforth abbreviated to NanK).

In bacteria, enzymes from two distantly related protein families carry out ATP-driven sugar phosphorylation (13): the glucokinase family (14) and the Repressor, Open reading frame, Kinase (ROK) family (15). Both families have the ribonuclease H-like protein fold, a sugar substrate-induced conformational change, ATP-binding DxGxT and GTGxG motifs, and a sugar-binding ExGH motif (13,15). The sugar substrate binding site is also somewhat conserved, with ligand-bound crystal structures showing five conserved sugar binding residues (Fig. 1B) (13). The key difference between the families is that ROK proteins have a Zn-binding motif (CxCGxxGCx(E/D)) that buttresses the ExGH motif histidine, placing it in the correct position for substrate binding (Fig. 1C). In contrast, the glucokinase family lack the Zn-binding motif and instead have an additional α-helix to structure the region (16).

We conducted a sequence alignment of NanK enzymes from bacteria, which are expected to be part of the ROK family (13). Curiously, we found that the majority of Gram-positive bacterial NanK sequences lack the Zn-binding motif and the α-helix used in the glucokinase family (Supporting Information 1 and Fig. S1). This is best shown in an alignment of representative NanK sequences (Fig. S2), which reveals that the cysteine residues usually involved in forming the Zn-binding motif are absent in sequences of Gram-positive S. aureus and S. mitis species, and the histidine of the ExGH motif is substituted with a tyrosine (ExGY) (17). This is surprising, as Zn-binding is required for activity in ROK proteins (15,18), is present throughout Gram-negative NanK isozymes (13,19), and the Zn-binding motif differentiates the ROK family from the glucokinase family (15).

Given that many Gram-positive NanK enzymes have been traditionally annotated as ROK enzymes, but are missing the essential Zn-binding motif, and instead have a tyrosine in place of histidine from the sugar-binding ExGH motif, we studied how Gram-positive S. aureus NanK (SaNanK) functions without these canonical features. Here we report the first functional and structural study of a Gram-positive NanK. We discover that the enzyme is active without Zn-binding, has a unique structured arginine π-stack in a position equivalent to the ROK Zn-binding motif, or glucokinase family α-helix, and that the tyrosine in the ExGY motif provides specificity for the N-acetylmannosamine substrate.

Results and Discussion

SaNanK N-acetylmannosamine kinase catalytic function

We first verified whether SaNanK was a N-acetylmannosamine kinase using an established assay for measuring initial rate data (19). The
enzyme is active with N-acetylmannosamine and ATP. Initial rate data, where both acetylmannosamine and ATP concentrations are varied, are best fitted with the ternary complex kinetic model (Fig. 2A), with an \( R^2 = 0.999 \) (see Experimental procedures for a discussion on how this model was chosen). This gives the following kinetic parameters: \( K_M^{N\text{-acetylmannosamine}} = 0.30 \text{ mM}, \) \( K_M^{N\text{-acetylmannosamine}} = 0.73 \text{ mM}, \) and \( k_{cat} = 262.6 \text{ s}^{-1} \) \( (V_{max} = 497 \mu\text{mol min}^{-1} \text{ mg}^{-1}) \) (Table 1). The \( k_{cat}/K_M \) for \( N\)-acetylmannosamine is 870 mM\(^{-1}\) s\(^{-1}\), which is on the same order as the only other \textit{bona fide} bacterial NanK enzyme characterised (from \textit{E. coli}) (19), and other ROK kinases (19-23). Thus, \( Sa\text{NanK} \) is catalytically active and kinetically comparable with other ROK kinases, despite missing conserved and defining active site motifs thought to be important for function.

Three ROK \textit{Streptomyces} glucokinases have been reported to have a compulsory-ordered mechanism, where glucose binds the active site before ATP (16,24). When the initial rate data is presented as double reciprocal plots the pattern provides information on whether the ternary complex kinetic mechanism is of the random- or compulsory-ordered type with respect to substrate binding (25). The plots for \( Sa\text{NanK} \) are consistent with a random-ordered ternary complex for catalysis (Fig. S5). These results demonstrate that \( Sa\text{NanK} \) binds substrate differently than \textit{Streptomyces} ROK glucokinases, which presumably have a structural feature that prevents ATP from binding first.

Glucose kinase activity is thought to be the ancestral function for the ROK family (13). Glucose differs from \( N\)-acetylmannosamine in that a equatorial (\( R \))-hydroxyl at the C2 position is substituted with an axial (\( S \))-N-acetyl group. To probe substrate specificity for the C2 position, we compared the catalytic function with \( N\)-acetylmannosamine against glucose and \( N\)-acetylglucosamine, which are C2 substrate analogues. The apparent \( K_M \) (\( K_M^{\text{pp}} \)) of \( N\)-acetylmannosamine (260 \( \mu \text{M} \)) is comparable with \( N\)-acetylmannosamine (300 \( \mu \text{M} \)) (Fig. 2B). However, the \( k_{cat} \) (23 \( \text{s}^{-1} \)) is 11-fold lower. The \( K_M^{\text{pp}} \) for glucose (15 \( \mu \text{M} \), Fig. 2C) is 60-fold higher than the \( N\)-acetyl substrates, highlighting that the C2 \( N\)-acetyl group is important for binding to the enzyme. In ROK glucokinases, the C2 position is bound by the ExGH motif histidine (13), which in \( Sa\text{NanK} \) is changed to tyrosine and represents an uncharacterised change within the ROK protein family. This result demonstrates that the \( N\)-acetyl substrate group is important for substrate binding and consequently the residues of the enzyme that coordinate the \( N\)-acetyl are important for substrate specificity.

Overall, we demonstrate that \( Sa\text{NanK} \) is a \textit{bona fide} \( N\)-acetylmannosamine kinase with catalytic constants comparable to canonical ROK kinases that have the ExGH and Zn-binding motif. We show that \( Sa\text{NanK} \) catalysis has a random-ordered ternary mechanism, in contrast to \textit{Streptomyces} glucokinases, which bind glucose first. The difference in kinetic mechanism suggests that \( Sa\text{NanK} \) has novel structural features, which may be related to the altered protein sequence. We also demonstrate that the C2 \( N\)-acetyl group of the substrate is important for substrate binding.

**Structure of \( Sa\text{NanK} \)**

To investigate the structural implications of the missing canonical ROK motifs and the altered kinetic mechanism relative to other glucokinases, we solved the crystal and solution structure of \( Sa\text{NanK} \) in the ligand-free, \( N\)-acetylmannosamine-bound, and \( N\)-acetylglucosamine-bound forms. The ligand-free structure was solved to 2.33 \( \AA \) resolution. Since we were unable to solve the structure using molecular replacement, selenomethionine-substituted enzyme was crystallised as previously described (17) and initial phases were determined using \textit{SHELX} (26). The asymmetric unit contains four NanK monomers where chains A, B and C are complete, but chain D has three regions where electron density is ambiguous and cannot be modelled (Gln28-Pro33, Pro82-Pro85, Asn142-Gln144). \( N\)-Acetylmannosamine-bound and \( N\)-acetylglucosamine-bound \( Sa\text{NanK} \) structures were each solved at 2.20 \( \AA \) resolution, using the ligand-free structure for molecular replacement. Electron density was present and distinguishable for all residues, except Met1. The data collection and refinement statistics are given in Table 1.

**The \( Sa\text{NanK} \) monomer**

The \( Sa\text{NanK} \) monomer features two domains, an N-terminal \( \alpha/\beta \) domain (Fig. 3A, red) and a larger \( \alpha+\beta \) domain (Fig. 3A, pink) that together form the ribonuclease H-like fold. The domains are orientated such that the monomer resembles an open clam, with the two separated domains being joined at a single hinge region (Fig. 3A, green). The active site is situated between the
two ‘half-shell’ domains, where residues from both domains are used to bind substrate and support catalysis. The structure is consistent with canonical ROK kinases, with the secondary structure regions matching *E. coli* NanK (Fig. S6) and comparison with TM-align (27) provides a structure match score of 84.4% and 86.6% with *E. coli* NanK (PDB: 2AA4) and *Streptomyces griseus* glucokinase (PDB: 3VGK), respectively.

The active sites of ROK kinases are generally well-conserved (13). Mapping the sequence alignment to the structure demonstrates that it is the most highly-conserved area of *Sa*NanK (Fig. 3B). However, from our sequence analysis, there is a major change to the region with the absence of a Zn-binding motif, which in canonical ROK partially encloses the active site from the sugar side (PDB: 3VGL). In *Sa*NanK, the region equivalent to the Zn-binding motif is shorter (13 vs. 20 residues) and is folded away from the active site area, interacting with residues of the large α+β domain.

**Sa*NanK is a dimer, consistent with other NanK enzymes**

To determine the relevant oligomeric state in solution, sedimentation velocity analytical ultracentrifugation and small angle X-ray scattering experiments were conducted. Both experiments demonstrate that *Sa*NanK is a dimer (across the concentration range tested).

Sedimentation velocity data were fit with the c(s) and c(M) models using SEDFIT (28), which give a single peak corresponding to 3.95 S and 62 kDa (Fig. 4A); thus, ligand-free *Sa*NanK is a dimer in solution given a monomeric mass of 31.7 kDa calculated from the sequence. A single peak is evident at each concentration, suggesting that the equilibrium constant for dimer dissociation (*K*<sub>D</sub> <sup>2,1</sup>) is very low. Small angle X-ray scattering data are also consistent with a dimer. The theoretical scatter for the ligand-free *Sa*NanK canonical dimer formed in the crystal structure (generated by CRYSTOL from the ATSAS package (29)) fitted closely to the scattering data (*X*<sup>2</sup> = 1.7) (Fig. 4B). We also tested a possible dimer formed by a secondary interface in the asymmetric unit (as defined by *PDBe PISA* (30), and the full asymmetric unit of the ligand-free structure (two dimers) (Fig. S7), both of which fitted poorly to the scattering data. The dimeric configuration is consistent with other NanK crystal structures (19,31,32) and MALDI-TOF mass spectrometry analysis of (ROK) glucokinase from *Bacillus subtilis* (15).

From the crystal structure, the dimeric configuration orients the active sites facing outward on opposite sides of the assembly, while the hinge regions are near the centre (Fig 4C). Residues from the large α+β domain form the dimerisation interface. This configuration is consistent with *E. coli*, *Fusobacterium nucleatum* and human NanK (19,31,32). *PDBe PISA* analysis indicates that the *Sa*NanK interface has a surface area of 1533.8 Å<sup>2</sup>, featuring five salt bridges and 18 hydrogen bonds. The residues identified by *PDBe PISA* contribute to three positive and three negatively charged regions that charge complement the opposing monomer (Fig. 4D).

In summary, we demonstrate that *Sa*NanK is a dimer. The dimeric interface consists of three complementary positive and negative patches on the surface of large α+β domain.

**Substrate induced changes in conformation**

A conformational change upon sugar binding, whereby the lobes of the enzyme close over the substrate, has been demonstrated in both the ROK and glucokinase families (14,16,31). Whether *Sa*NanK undergoes a similar conformational change was determined by comparing the sugar substrate bound and ligand-free X-ray crystal structures. In the N-acetylmannosamine-bound *Sa*NanK structure the two domains are shifted 7 Å inwards and close around the substrate, like the shells of a clam enclosing a pearl (Fig. 5A). As a dimer, the movement occurs mainly in the N-terminal α/β domain, pivoting at the hinge region, as the large α+β domain is set by the dimeric interface. Overall, the conformational change of *Sa*NanK is consistent with other ROK kinases (14,16,31).

The purpose of a conformational change is hypothesised to ensure that ATP hydrolysis occurs only when sugar substrate is present, providing efficient catalysis (31). To investigate, the catalytic residues identified from the sequence alignment (Fig. S2) were compared between ligand-free and N-acetylmannosamine-bound structures. In the catalytic mechanism for sugar kinases, ATP is primed for nucleophilic attack at the γ-phosphate by electron withdrawal through the β-phosphate, which then undergoes nucleophilic attack by the sugar (31). For *Sa*NanK, electron withdrawal likely occurs via Asp7 binding Mg<sup>2+</sup> associated with the ATP β-phosphate, given its proximity, while Thr11 and
Thr134 hydrogen bond with the other side of the β-phosphate group. Alignment of Mg-ADP from the human NanK structure demonstrates that when N-acetylmannosamine is bound, Thr134 is shifted towards the expected β-phosphate position (Fig 5B, pink) and both Asp7 and Thr11, which are part of the N-terminal α/β domain, make a large shift towards the ATP position (Fig 5B, red). Importantly, this shows that Asp7, Thr11 and Thr134 are in position to provide electron withdrawal in the N-acetylmannosamine-bound structure and not the ligand-free structure. In short, this is consistent with a conformational change that ensures ATP is only hydrolysed in the presence of substrate (discussed in (31)).

As conformational change is important for catalysis in ROK kinases (discussed above), the N-acetylglucosamine and N-acetylmannosamine bound structures were compared to rationalise the lower catalytic rates with N-acetylglucosamine. The structure of N-acetylglucosamine-bound SaNanK is similar to the N-acetylmannosamine bound structure but appears to be slightly more open (Fig. S8), with an average RMSD for the four chains of 0.42 Å using 262-265 of the 285 residues (PyMOL). This alters the distances between catalytic residues Thr11 and Thr134, which provide electron stabilisation to the β-phosphate of ATP and provides a possible explanation for lower catalytic rates with N-acetylglucosamine.

We then examined whether conformational change explains why the enzyme operates via an ordered or random kinetic mechanism. To do this we compared the ligand-free and sugar-bound structures of SaNanK, which has a random ordered kinetic mechanism, with S. griseus glucokinase (PDB: 3VGK & 3VGM), which has a compulsory ordered kinetic mechanism, where glucose binds first and then ATP binds (16). Both SaNanK and S. griseus glucokinase undergo a similar overall conformational shift, pivoting at the hinge region in response to sugar-binding and the ATP pocket is essentially unchanged (Fig. S9). Therefore, conformational change does not seem to explain why some ROK kinases have a random kinetic mechanism, while others have a compulsory ordered kinetic mechanism.

To test whether the conformational change seen in the crystal structure also occurs in solution, small angle X-ray scattering was used. The scattering profile with and without N-acetylmannosamine were individually compared with the theoretical scatter of the crystal structures with and without N-acetylmannosamine using CRYSOL (33). The scattering data from the ligand-free SaNanK sample closely matches the ligand-free SaNanK crystal structure, but not the N-acetylmannosamine-bound crystal structure, (reduced $\chi^2$ of 1.7 vs. 4.9) (Fig. 4B). In contrast, the scattering data for SaNanK in the presence of N-acetylmannosamine (5 mM) closely matches the N-acetylmannosamine-bound crystal structure, but not the ligand-free SaNanK crystal structure (reduced $\chi^2$ of 1.9 vs. 9.9) (Fig. 5C). This demonstrates that the crystal structures match the solution structures and the enzyme closes around N-acetylmannosamine. The condensing of the N-acetylmannosamine solution structure is further supported by a smaller radius of gyration calculated from the overall scattering intensity ($R_g = 26.9$ Å vs 27.5 Å) (Fig. 5D).

To summarise, SaNanK undergoes an N-acetylmannosamine induced conformational change adopting a closed conformation. This ensures ATP hydrolysis only occurs in the presence of sugar substrate. In support, the catalytic residues (Asp7, Thr11 and Thr134) that withdraw electrons from the β-phosphate group are only in an active configuration in the closed conformation. Adherence to the closed and open conformations, with and without ligand respectively, is demonstrated in solution by small angle X-ray scattering.

### The SaNanK active site uses tyrosine to bind the substrate C2 position

Five sugar substrate binding residues are reported to be well conserved in ROK kinases (13) and conservation was investigated in SaNanK. Four of the five residues are conserved, including Asn107, Asp108, Glu157 and Glu172, whereas Tyr160 is a non-conservative change and replaces the histidine in the canonical ExGH motif (discussed later). The C1 hydroxyl of N-acetylmannosamine is bound by Glu172 and the Glu157 primarily binds C3, but also binds the C2 position N-acetyl moiety. Both residues are part of the larger α+β domain (Fig. 6A magenta). Asn107 binds the C4 hydroxyl and Asp108 binds C6 (Fig. 6A). These residues border the N-terminal α/β domain and the hinge region, where the conserved substrate binding translates to the conformation change between the ligand-free and N-acetylmannosamine-bound structures. In addition to substrate binding, the conserved Asp108 that binds the C6 position is also expected to feature in...
the catalytic mechanism by deprotonating the hydroxyl and increasing nucleophilicity (34). Consistent with the mechanism, Asp108 is predicted to be deprotonated in the ligand-free structure (pKa = 6.03), but protonated in the N-acetylmannosamine-bound structure (pKa = 9.14), using PDB2PQR (35) at pH 7.

The structural role of Tyr160 in the ExGY motif of SaNanK is a non-conservative substitution of the Zn-binding histidine in the ExGH motif seen in canonical ROK kinases. Indeed, tyrosine at this position is conserved in other Gram-positive NanK lacking the Zn-binding motif (Fig. S1). The hydroxyl of SaNanK Tyr160 binds the C2 N-acetyl group of N-acetylmannosamine (Fig. 6A). This is analogous to ROK glucokinase, where the equivalent ExGH motif histidine binds the C2 hydroxyl of glucose (16). However, glucose is much smaller and the ExGH motif histidine also binds Zn. Zn-binding NanK enzymes utilise the ExGH motif histidine to bind the C1 hydroxyl and the larger N-acetyl substrate is bound from residues on the other side of the active site (31). A comparison of substrate binding is presented in Fig. 6B. In contrast to histidine found in the Zn-binding structures, SaNanK Tyr160 tilts away from the active site, increasing the size of the active site, and hydrogen bonds with the N-acetyl group. Interestingly, N-acetylmannosamine binds in a ‘boat’ conformation and the N-acetyl group is in a pseudo-equatorial position (Fig. 6B, S10, S11). It is unknown whether these observations are typical for NanK without the Zn-binding motif, although our sequence alignment suggests that tyrosine is consistently present at the position.

Our earlier kinetic analysis revealed that the C2 N-acetyl group is important for binding, with the \( K_M \) of both N-acetylmannosamine and N-acetylglucosamine (0.30 mM and 0.26 mM, respectively) being much lower than glucose (15 mM), which only differs at the C2 position. To investigate further, the N-acetylglucosamine-bound and N-acetylmannosamine-bound structures were compared. Both substrates are bound at the C1, C3, C4 and C6 hydroxyls by Glu172, Glu157, Glu107 and Asp108, respectively, (Fig. 6A and 6C). These positions are conserved with S. griseus glucokinase and glucose is expected to bind with SaNanK in a similar manner. At the C2 position, the N-acetyl groups are both in an equatorial position, despite being enantiomers. N-acetylmannosamine adopts a ‘boat’ conformation with the carbonyl pointing ‘down’ and the N-acetyl is positioned to bind three residues, Gly81 to the carbonyl, Glu157 to both the carbonyl and amine, and Tyr160 to carbonyl (Fig. 6A). In contrast, N-acetylglucosamine adopts a ‘chair’ conformation with carbonyl pointing ‘up’ and the N-acetyl group is positioned to bind two residues only, Gly81 to the carbonyl and Glu157 to the amine (Fig. 6C). The comparison reveals that SaNanK the active site is structurally optimised to bind N-acetylmannosamine, over N-acetylglucosamine and glucose.

Next, we tested the role of the Tyr160 hydroxyl in substrate specificity by producing substituted enzyme SaNanK-Tyr160Phe and comparing its kinetic profile (Table 3 and Fig. 6D). For N-acetylmannosamine, the specificity constant for the wild-type \( (k_{cat}/K_M = 870 \text{ mM}^{-1} \text{ s}^{-1}) \) is greater than the Tyr160Phe substituted enzyme \( (k_{cat}/K_M = 170 \text{ mM}^{-1} \text{ s}^{-1}) \). This is consistent with the participation of the Tyr160 hydroxyl in substrate binding for wild-type SaNanK. For N-acetylglucosamine, the specificity constant for the wild-type \( (k_{cat}/K_M = 90 \text{ mM}^{-1} \text{ s}^{-1}) \) is lower than the Tyr160Phe substituted enzyme \( (k_{cat}/K_M = 390 \text{ mM}^{-1} \text{ s}^{-1}) \). This suggests that the Tyr160 hydroxyl may have a role in perturbing N-acetylglucosamine binding. In support of this, Tyr160 is displaced by 1 Å, compared to the ligand-free and N-acetylmannosamine-bound structures, indicating that the residue sterically hinders N-acetylglucosamine binding. Together, the results demonstrate that the Tyr160 hydroxyl is important for specificity towards the pathway substrate, with a 5:1 preference for N-acetylmannosamine with tyrosine, compared to a 2:1 preference for N-acetylglucosamine when replaced with phenylalanine. Controls with Tyr160Ala substituted enzyme (Table 3) were similar to Tyr160Phe and further support that the tyrosine hydroxyl is important for N-acetylmannosamine specificity.

Without the Zn-bound ExGH motif histidine, SaNanK binds the N-acetyl group of N-acetylmannosamine with tyrosine, which is generally conserved in Gram-positive NanK without the Zn-binding motif. This is different to Zn-binding NanK enzymes, which bind the N-acetyl group using residues on the other side of the active site (31). The tyrosine residue is important for substrate recognition and when replaced with phenylalanine, substrate preference shifts to N-acetylglucosamine.
The ROK Zn-binding motif is replaced by a stabilising arginine stack

SaNanK does not have the highly-conserved ROK Zn-binding motif, however the significance of this is unknown as the purpose of the motif is not well-understood (13). To identify structural differences, SaNanK was compared with a ROK and a non-ROK sugar kinase. In ROK S. griseus glucokinase, the Zn-binding region encompasses 20 residues, starting from the Zn-binding ExGH motif His157 to the end of the motif, CxCGxxGCx(E/D) (residues 167-176, PDB: 3VGK). The ExGH motif histidine and the Zn-motif Glu/Asp bind to the sugar, with the central residues forming a loop that wraps around Zn (Fig. 1C) enclosing the active site (Fig. 7A, green). For SaNanK ROK without the Zn-motif, the equivalent region between the sugar-binding Tyr160 and Glu172 (13 residues) forms a loop that associates with the large α+β domain and does not occlude the sugar active site (Fig. 7A, red). In non-ROK E. coli glucokinase, the equivalent region is 28 residues long (His160-Glu187) and features highly charged residues as part of the dimerisation interface (14). This does not occlude the sugar active site (Fig. 7A, yellow) in a position similar to the SaNanK region. The observations suggest that the absence of Zn-binding corresponds with an open sugar active site and the presence of Zn-binding corresponds with a closed active site.

The absence or presence of the Zn-motif may determine the order of substrate binding. We propose that SaNanK has a random-order kinetic model because the absence of the Zn-motif provides a secondary substrate entry point, whereas in the compulsory-order S. griseus glucokinase there is a narrow active site with just a single entry and glucose must access the pocket at the bottom before ATP binds. Kinetics assays demonstrate that SaNanK uses a random-order ternary complex for catalysis, whereas canonical ROK S. griseus glucokinase is characterised as a compulsory-ordered complex that requires glucose to bind before ATP (16). Initially, conformational change was investigated as a method of conferring order (discussed above), but comparisons of the enzyme structures failed to identify a differentiating feature in ATP-binding site and it is difficult to attribute the difference in mechanism to conformational change. Compulsory-ordered S. griseus glucokinase has a narrow active site with a single opening near the ATP-binding pocket (Fig. 7A). Glucose binds at the bottom of the active site and ATP binding would obstruct glucose binding, thus providing an explanation of why glucose must bind before ATP. In contrast, SaNanK has a secondary opening near the active site that N-acetylmannosamine can access irrespective of ATP binding providing for a random-ordered kinetic mechanism.

ROK Zn-binding has been demonstrated elsewhere to be necessary for activity (15,18), and Zn coordination is hypothesised to organise three nearby active site residues (16). The organisation of equivalent residues (Glu157, Tyr160 and Glu172) were examined in SaNanK to investigate why the enzyme is active without Zn-binding. Glu157, Tyr160 and Glu172 are well-organised in the ligand-free structure with lower B-factors (17.5, 13.8, 18.8) than the model average (37.7), despite little secondary structure organisation in the region. The nearby region, equivalent to the ROK Zn-binding region, was examined for potential stabilising features. Interestingly, the region features two stacked arginine residues (Arg164, Arg174), ionically coordinated by three negatively charged residues (Glu167, Asp219, Glu222), with two of the negative residues belonging to a large α-helix that anchors the arginine stack (Fig. 7B). Potentially, this five-coordinate feature stabilises the region and organises the active site residues, serving the hypothesised role of four-coordinate Zn-binding in canonical ROK.

Re-examination of Gram-positive NanK sequence alignments reveals that the arginine stacking residues are novel within the Staphylococcus genus and Arg164, which is coordinated by four other residues, is not conserved elsewhere (Fig. S1). Arg164 was substituted with alanine and we test the effect of this change on thermal stability to test for structural stabilisation, and kinetic assays to test whether the residue is important for active site organisation (a hypothesised role of the equivalent Zn-binding region (16)). SaNanK-Arg164Ala was not recoverable in the soluble fraction and attempts to improve solubility by altering the expression temperature and length were unsuccessful. This suggests the enzyme is potentially unstable without the positive charge or hydrophobic stacking supplied by arginine. However, SaNanK-Arg164Lys was successfully purified. Thermal stability analysis reveals that SaNanK-Arg164Lys has an average $T_m$ of 35.4 °C, which is 4.1 °C lower than SaNanK (Fig. 7C), and in conjunction with insolvency of SaNanK-Arg164Ala, this indicates that arginine interaction is important for
stabilisation. Kinetic assays show that the substituted enzyme is catalytically compromised \((V_{\text{max}} = 6.1 \pm 0.3 \ \mu\text{mol/min/mg}, k_{\text{cat}} = 3.2 \pm 0.2 \ \text{s}^{-1}, K_M = 0.26 \pm 0.01 \ \text{mM})\) with approximately a 100-fold decrease in activity compared to wild type \((k_{\text{cat}} = 262.6 \ \text{s}^{-1})\). This is consistent with arginine stacking contributing to organise the nearby active site residues. This is a hypothesised role of the ROK Zn-binding region, which is equivalently positioned to the SaNanK arginine stack, and we propose that both coordinating features provide stabilisation and organisation.

The absence of the Zn-binding motif increases the potential of SaNanK as a drug target. Elsewhere, it has been demonstrated that the bacterial sialic acid catabolic pathway is a viable target to lower infectivity and increase antibiotic efficacy \((1,3,4,9,10)\). Targeting the pathway is only useful if toxic interaction with host sialic pathway enzymes can be avoided. The NanK component of human bifunctional UDP-\(N\)-acetylglucosamine \(2\)-epimerase/\(N\)-acetylmannosamine kinase is a ROK family protein but, unlike SaNanK, it retains the canonical Zn-binding motif \((31)\). The structure of the human enzyme (PDB: 2YHY) shows that bound \(N\)-acetylmannosamine is enclosed by the Zn-binding motif and the \(N\)-acetyl group associates in the axial position. Whereas, our characterisation demonstrates that SaNanK is open at the \(N\)-acetylmannosamine end of the active site (Fig. 7A). Therefore, we propose that synthesised glucose or mannose C2 (or C1) derivatives can be made to target SaNanK and not the human bifunctional enzyme, where the Zn-binding motif occludes larger derivative.

Conclusions

Gram-positive NanK enzymes are annotated as ROK enzymes, but many are missing the identifying ROK Zn-binding motif and have tyrosine in place of the histidine in the ExGH motif. Here we investigate the effect of these changes on the structure and function of SaNanK. We focus on defining the role of tyrosine that replaces ExGH motif histidine and provide insight into the presence or absence of Zn in ROK kinases.

We report the first functional and structural study of a Gram-positive NanK. The crystal and solution structures conform to the ribonuclease H-like fold present in ROK family proteins \((36)\) and we confirm that the enzyme is catalytically active as a kinase. Therefore, SaNanK has the properties expected from a ROK kinase. Investigation of the protein dynamics reveal the structure condenses with sugar substrate in the X-ray crystal form, as reported for other ROK kinases \((16,31)\). For the first time, we confirm this in solution with small-angle X-ray scattering experiments.

Our sequence alignment reports that Gram-positive NanK without the Zn-binding motif have tyrosine in place of ExGH motif histidine. Structural and functional analysis reveals that tyrosine binds C2 \(N\)-acetyl of \(N\)-acetylmannosamine by tilting backwards from the active site. This is substantially different from Zn-binding NanK, where ExGH motif histidine binds C1 and is restrained in a forward-facing tilt by secondary binding of Zn. As ExGH motif histidine binds Zn, the use of tyrosine in Gram-positive NanK is certainly linked to the loss of Zn-binding. However, as Gram-positive NanK are highly divergent at a sequence level, it is difficult to know whether change of histidine from the ExGH motif or Zn-binding motif residues occurred first.

The role of the Zn-binding motif is not well-understood, therefore the significance of its absence in Gram-positive NanK is unknown. Our examination considered the absence of Zn-binding in SaNanK and its presence in canonical ROK. A role hypothesised for Zn-binding is that it organises the active site residues for catalysis \((16)\). We find a possibly equivalent coordinating feature in SaNanK—an arginine stack that is novel to \textit{Staphylococcus}. Loss of this feature in SaNanK lowered thermal stability and significantly lowered catalytic rates, consistent with the hypothesis that it is a feature that provides structural stabilisation and active site coordination, as opposed to being provided by that Zn-binding in other ROK kinases. Our structural comparison also revealed that absence of the motif provides an opening at the sugar substrate active site. This is beneficial for the active site access of larger substrates, such as \(N\)-acetylmannosamine in SaNanK and potentially, disaccharide cellobiose kinases \((23)\). Whereas Zn-binding in \textit{E. coli} NanK, requires increased hinge flexibility for \(N\)-acetylmannosamine association \((19)\). We propose that the ROK Zn-binding motif is an adaptation that excludes large substrates, consistent with ROK repressors and kinases generally functioning with small sugars \((13)\) that may also contribute to compulsory-ordered catalysis for pathway regulation \((16,24)\).

Finally, given the proximity of the arginine stack and ExGY motif, we propose that this is a
unique substructure to target with inhibitory compounds that avoid off target effects with the human homologue, bifunctional NanK.

**Experimental procedures**

**Bioinformatics**

To provide a root for the phylogenetic tree of NanK protein sequences, sequences for glucokinase, fructokinase and N-acetylglucosamine kinase enzymes that were closely related to the canonical ROK sequence were added to the alignment. To select the root sequences, the ROK Hidden Markov Model (HMM) sequence from the Pfam database (https://pfam.xfam.org/family/PF00480/hmm) was entered into pBLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), alongside the search terms “glucokinase”, “fructokinase” or “N-acetylglucosamine kinase.” The top three sequence results (from unique genera) were included in the alignment. NanK protein sequences were searched for by inputting the SaNanK sequence, ROK HMM sequence and *Escherichia coli* NanK sequence, alongside the search term “N-acetylmannosamine kinase” into the tBLASTn database. The first NanK sequence for each unique genus were added to the alignment. Additionally, sequences that had a different variation of ExGH motif or Zn-binding residues, were included. To verify sequences were annotated correctly, only sequences clustered with Nan genes were included.

**Cloning, expression and purification**

SaNanK-Arg164Ala, -Arg164Lys and Tyr160Ala were constructed by Genscript based on variation of *S. aureus* nanK (Accession No. YP_498886.1) codon optimised for expression in *E. coli*. SaNanK-Arg164Ala was synthesised *de novo* and inserted into a pET30ΔSE plasmid between *NdeI* and *HindIII* restriction sites. SaNanK-Arg164Lys was then created by site-directed mutagenesis.

SaNanK-Tyr160Phe was obtained by in house site directed mutagenesis of the pET30ΔSE-SaNanK (17,37) using the infusion protocol (38), with forward primer (5’-A GTT GGG TTT TTA TTG TAT CGT CCA ACT GAA AA-3’) and reverse primer (5’-AA TAA AAA CCC AAC TTC ATT TGC CTT ATG A-3’). Constructs were verified through DNA sequencing by Macrogen (Korea).

The protocol for expression and purification of wild type protein has previously been reported (17). Briefly, *E. coli* BL21 (DE3) cells were transformed with pET30ΔSE/NanK, then cultured in Luria-Broth containing kanamycin (30 µg ml⁻¹) at 37 °C, 180 rev min⁻¹ until an OD₆₀₀ of 0.6 was reached. Expression was induced with isopropyl β-d-1-thiogalactopyranoside at a final concentration of 1 mM and incubated overnight at 26 °C. Purification was conducted at 4 °C. Cells were harvested at 6,000 g for 10 min using a Thermo Sorvall RC-6-Plus centrifuge and resuspended in 20 mM Tris–HCl pH 8.0. The cells were lysed for 10 min (Hielscher UP200S Ultrasonic Processor at 70%, 0.5 s on/off cycle) and lyses containing soluble protein was extracted from the supernatant after centrifugation at 24,000 g. For anion exchange chromatography, the sample was applied to a 20 ml Q Sepharose anion exchange column (GE Healthcare) pre-equilibrated with lysis buffer, then eluted with an increasing concentration gradient of 20 mM Tris–HCl pH 8.0, 1 M NaCl. For hydrophobic exchange chromatography, ammonium sulfate was added to the eluted protein to a final concentration of 1 M and applied to 20 ml Phenyl Sepharose FF column (GE Healthcare) pre-equilibrated with 20 mM Tris–HCl pH 8.0, 1 M ammonium sulfate and eluted using an increasing concentration gradient of 20 mM Tris–HCl pH 8.0. Finally, size exclusion chromatography was conducted with a HiLoad 16/60 Superdex 200 column (GE Healthcare) using 20 mM Tris–HCl pH 8.5. Final purity was estimated using SDS page gels (Fig. S13). Exceptions to this procedure were as follows; selenomethionine substituted protein was produced using PASM-5052 autoinduction media (39) and expression trials of Arg164Ala mutants were conducted at 20 and 26 °C, for 4, 8 and 16 hours.

**Enzyme kinetics**

Initial rate data were recorded using a pyruvate kinase/lactate dehydrogenase coupled assay (19). NADH oxidation was monitored using a CARY 100 Bio UV-visible spectrophotometer (Agilent Technologies) based on an extinction coefficient of 6220 M⁻¹ cm⁻¹ at 340 nm (40). The reaction mixture incubated for 15 minutes at 25 °C, consisted of a pyruvate kinase/lactate dehydrogenase mix (10 µl, purchased from SIGMA SKU-P0294), purified wild type SaNanK 0.017 mg ml⁻¹, 536 nM (10 µl), phosphoenolpyruvate 50 mM (20 µl), NADH 8 mM (20 µl), ATP varied
concentration (20 μl), and kinetics buffer, Tris-HCl pH 8.5-22 mM, KCl 20 mM, MgCl₂ 5 mM (900 μl) – listed values are initial concentrations. The reaction was the initiated with sugar substrate (20 μl, at varied concentration), which was also incubated at 25 °C. To ensure that N-acetylmannosamine kinase concentration was directly proportional with the rate, it was checked that the coupling enzymes were in excess and that there was no background reaction. The enzyme appeared to be less stable when incubated at 30 °C or above, which was consistent with a differential scanning fluorimetry experiment demonstrating that the melting temperature of SaNanK is 39.5 °C (Fig. 7C). Moreover, the enzyme was deactivated when in Tris-HCl buffer at a pH of 8 or below. The data was processed using GraphPad Prism 6.0 (GraphPad Software, La Jolla California USA, www.graphpad.com). The model that best fitted the data [Ternary complex (Eq. 1) or substituted complex models (Eq. 2)] was determined by evaluating the AICc values (41) (ternary complex = 129.8, substituted complex = 203.4). Single substrate (apparent) kinetic parameters were determined using the Michaelis-Menten model.

Initial rate equations for ternary and substituted complexes (42).

\[
v = \frac{v_{ab}}{K_{ia}K_{mB} + K_{mBa} + K_{mAb} + ab} \quad \text{(Equation 1)}
\]

\[
v = \frac{v_{ab}}{K_{mBa} + K_{mAb} + ab} \quad \text{(Equation 2)}
\]

**Analytical Ultracentrifugation**

Analytical ultracentrifugation was performed using an XL-I analytical ultracentrifuge (Beckman Coulter) at 50,000 rev min⁻¹, 20 °C. Sedimentation velocity experiments were conducted with N-acetylmannosamine kinase at 0.3 0.6, and 0.9 mg ml⁻¹ (9.5, 18.9 and 28.4 μM), in Tris-HCl pH 8.5 20 mM, KCl 100 mM. Sample (380 μl) and reference buffer (400 μl) were loaded into 12 mm double-sector quartz cells with standard Epon 2-channel center-pieces and mounted in an An-50 Ti eight-hole rotor (Beckman). Radial absorbance was measured at 280 nm with a step size of 0.003 cm over 80 scans. SEDNTERP software was used to calculate values for solvent density, solvent viscosity and to estimate partial specific volume, based on the amino acid sequence (43). SEDFIT was then used to fit the data with continuous size distribution [c(s)] and continuous mass distribution [c(M)] models (28). Residuals were examined to review that noise was normally distributed and data points were checked for fit with curves (Fig. S4).

**Small Angle X-ray Scattering**

Small angle X-ray scattering was undertaken at the Australian Synchrotron on the SAXS/WAXS beamline equipped with a PILATUS 1 M detector (170 x 170 mm, effective pixel size 172 x 172 μm), as described by Griffin et al. (44). The x-ray wavelength provided was 1.0332 Å and the sample detector distance was set at 1600 mm, which produced a q range of 0.006-0.4 Å⁻¹. SaNanK (6 mg ml⁻¹, 189 μM, 50 μl) was injected onto an inline Superdex 200 5/150 GL (GE Healthcare) using a co-flow setup (Ryan et al. 2018). The column was equilibrated with 20 mM Tris-HCl pH 8.0, 100 mM NaCl and the radical scavengers 5% (v/v) glycerol and 0.1% (w/v) sodium azide, using a flow rate of 0.45 ml min⁻¹. Additionally, 5 mM N-acetylmannosamine was added to the equilibration buffer above for the sugar-bound sample. Over the course of the elution, scattering data were collected in 1-second exposures using a 1.0 mm glass capillary at 12 °C. To process the scattering data, ScatterBrain IDL (V 2.822, Australian Synchrotron Facility) was employed for initial data reduction, radial integration of individual frames and the conversion of 1D data files. Data was buffer-subtracted using CHROMIXS (45). The ATSAS software suite (version 2.8.0) was used for all further analysis (29), including calculation of the radius of gyration and pairwise distribution functions P(r). Theoretical scattering of the SaNanK crystal structures were calculated using CRYSOL (33).

**X-ray crystallography**

Crystallisation was performed using the sitting-drop vapour-diffusion method at 20 °C. For selenomethionine substituted SaNanK, drops were composed of enzyme 10 mg ml⁻¹ (200 nl) and reservoir solution (200 nl) containing sodium malonate dibasic monohydrate 0.2 M, 20% w/v PEG 3350 (Molecular Dimensions, PACT premier HT-96 Screen, condition E12). For N-acetylmannosamine and N-acetylglucosamine bound enzyme, drops contained SaNanK 10 mg ml⁻¹ and either N-acetylmannosamine or N-acetylglucosamine 10 mM (200 nl), plus a reservoir solution of 0.2 M L-arginine, 0.1 M sodium acetate, pH 5.0 in 8% w/v poly-γ-glutamic acid (200 nl) (Molecular Dimensions, PGA HT-96 Screen,
The crystals were cryo-protected by soaking in 15% ethylene glycol solution and reservoir solution prior to freezing in liquid nitrogen. The Australian Synchrotron MX1 and MX2 beamlines were used for data collection. Data collected for the selenomethionine substituted enzyme was processed using *iMosflm* (46) and *AIMLESS* (47). For enzyme with *N*-acetylmannosamine and *N*-acetylglucosamine, data was processed with *XDS* and *AIMLESS* (47). X-ray diffraction parameters are reported in Table 1.

Experimental phasing was conducted using anomalous scattering of *SaNanK* from selenium-derivatised methionine (6 sites per monomer). The diffraction data was phased using single anomalous diffraction (SAD) processed by *SHELXC/D/E*, including automated model building (26). Electron density of the final resolved phases are demonstrated in Fig. S3. Phases of *N*-acetylmannosamine and *N*-acetylglucosamine-bound *SaNanK* were calculated using the ligand-free *SaNanK* structure as a molecular replacement model with *MOLREP* (48). Models were iteratively refined and built, using *REFMAC5* and *COOT* (49,50). Finalisation and model validation was conducted using *phenix.refine* and *MOLPROBITY* (51,52).

Attempts to solve the phases of ligand-free non-selenomethionine substituted *SaNanK* using molecular replacement were unsuccessful. Search models for *SaNanK* were selected based on the highest sequence identity: 2GUP, 3VOV, 3VGK and 4HTL (identity = 28.0-24.6%). Additionally, two NanK structures were also trialled, 2YHY and 2AA4 (identity = 21.9 - 21.4%). The models were split into monomeric units and processed with *CHAINSAW*, to generate the search model, and molecular replacement was attempted using *MOLREP* and *PHASER* (48,53). We also tried partial models and lower packing restraints (54), but these were also unsuccessful.
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Conflict of interest: The authors declare that they have no conflicts of interest.
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Tables

**Table 1. Kinetic parameters for wild type SaNanK.** The \( V_{\text{max}} \), \( k_{\text{cat}} \) and \( K_M \) were calculated in GraphPad Prism. The error is the standard error of the fit. \( N \)-Acetylmannosamine parameters were produced from global fits by varying ATP.

| Parameter               | \( N \)-Acetylmannosamine | Glucose* | \( N \)-Acetylglucosamine* |
|-------------------------|-----------------------------|----------|-----------------------------|
| \( V_{\text{max}} \) (\( \mu \)mol/min/mg) | 497 ±7                      | 114 ±3   | 44.4 ±0.3                   |
| \( k_{\text{cat}} \) (s\(^{-1}\))       | 263 ±3                      | 60 ±1    | 23.4 ±0.2                   |
| \( K_M \) (mM)           | 0.30 ±0.01                  | 15 ±1    | 0.260 ±0.005                |
| \( k_{\text{cat}}/K_M \) (s\(^{-1}\)/mM) | 871 ±34                     | 4.0 ±0.3 | 90 ±3                       |
| ATP \( K_M \) (mM)       | 0.73 ±0.02                  |          |                             |

* Glucose and \( N \)-acetylglucosamine are apparent parameters, using saturating ATP concentration.
Table 2 | Data collection and refinement statistics.

| PDB   | Ligand-free            | N-Acetylmannosamine-bound | N-Acetylglucosamine-bound |
|-------|------------------------|---------------------------|---------------------------|
|       | 6Q26                   | 6Q27                      | 6Q28                      |
| Wavelength | 0.9724               | 0.954                     | 0.954                     |
| Resolution range | 48.736-2.328 (2.412-2.328) | 44.556-2.200 (2.279 2.200) | 47.88-2.2 (2.279-2.200) |
| Space group | C 2 2 2 1             | P 2 1                     | P 2 1                     |
| Unit cell | 116.7, 135.2, 175.3   | 47.2, 101.4, 133.2; 90.15 | 47.9, 102.0, 133.3; 90.14 |
| Total reflections | 867,330 (83,021)      | 124,343 (11,758)          | 255,819 (25,619)          |
| Unique reflections | 59,453 (5,864)       | 63,756 (6,331)            | 64,898 (6,477)            |
|Multiplicity | 14.6 (14.2)         | 2.0 (1.9)                 | 3.9 (4.0)                 |
|Completeness (%) | 99.9 (99.4)         | 99.9 (99.9)               | 99.8 (99.5)               |
|Mean I/sigma(I) | 20.0 (1.9)          | 4.9 (1.0)                 | 5.3 (0.9)                 |
|Wilson B-factor | 30.98               | 43.34                     | 30.46                     |
| $R_{merge}$ | 0.237 (1.557)       | 0.083 (0.523)             | 0.315 (0.692)             |
| $R_{meas}$  | 0.245 (1.615)       | 0.117 (0.740)             | 0.368 (0.804)             |
| $R_{pim}$   | 0.064 (0.424)       | 0.083 (0.523)             | 0.187 (0.405)             |
|CC1/2       | 0.994 (0.671)       | 0.988 (0.704)             | 0.881 (0.701)             |
|Reflections used in refinement | 59447 (5864)   | 63746 (6330)              | 64886 (6477)              |
|Reflections used for R-free | 6036 (622)     | 6340 (603)                | 3202 (315)                |
| $R_{work}$  | 0.1973 (0.2711)     | 0.1940 (0.3098)           | 0.2198 (0.3312)           |
| $R_{free}$  | 0.2400 (0.3284)     | 0.2324 (0.3362)           | 0.2400 (0.3480)           |
|Number of non-hydrogen atoms | 9103               | 8989                      | 9230                      |
| Macromolecules | 8808               | 8840                      | 8896                      |
| Ligands     | N/A                  | 60                        | 60                        |
| Solvent     | 295                  | 89                        | 274                       |
| Protein residues | 1131             | 1140                      | 1144                      |
| RMS (bonds) | 0.009                | 0.009                     | 0.016                     |
| RMS (angles) | 1.30               | 1.27                      | 1.77                      |
|Ramachandran favored, allowed, | 98.3, 1.7, 0    | 98.1, 1.9, 0              | 98.2, 1.7, 0.1            |
|Rotamer outliers (%) | 0                 | 0.64                      | 0.53                      |
|Average B-factor (Å²) | 37.1              | 51.9                      | 38.3                      |
| macromolecules | 37.3               | 51.8                      | 37.9                      |
|ligands     | N/A                  | 69.8                      | 55.1                      |
| solvent    | 32.3                 | 55.6                      | 48.3                      |
| Number of TLS groups | 15                | 16                        | 24                        |
|Anomalous completeness (%) | 99.9 (98.3) | 99.9 (98.3) | 99.9 (98.3) |
|Anomalous multiplicity | 7.6 (7.2)  | 7.6 (7.2)                 | 7.6 (7.2)                 |
**Table 3. Kinetic parameters for SaNanK substituted at Tyr160.** Without the Tyr160 hydroxyl group, SaNanK-Tyr160Phe and SaNanK have a higher enzyme specificity ($k_{\text{cat}}/K_M$) with N-acetylglucosamine than for N-acetylmannosamine, the pathway substrate. The $V_{\text{max}}$, $k_{\text{cat}}$ and $K_M$ were calculated in GraphPad Prism. The error is the standard error of the fit. Values are apparent parameters, using saturating ATP concentration.

|                          | $N$-Acetylmannosamine | $N$-Acetylglucosamine |
|--------------------------|-----------------------|-----------------------|
|                          | $Sa$NanK-Tyr160Phe    | $Sa$NanK-Tyr160Ala    |
| $V_{\text{max}}$ (µmol/min/mg) | 408 ±5                | 422 ±8                |
| $k_{\text{cat}}$ (s$^{-1}$)    | 216 ±3                | 223 ±4                |
| $K_M$ (mM)               | 1.27 ±0.1             | 1.34 ±0.1             |
| $k_{\text{cat}}/K_M$ (s$^{-1}$/mM) | 171 ±13              | 167 ±13              |
Figures

A) A schematic of sugar flow through the bacterial sialic acid catabolism pathway. NanK catalyses the conversion of N-acetylmannosamine and ATP to form N-acetylmannosamine-6-phosphate and ADP. This proposed pathway is based on experimental evidence from *S. aureus* (4), *B. subtilis* and *E. coli* (55), as well data from KEGG genome data (56). For clarity, substrate and enzyme names are abbreviated. Enzymes: Sialic acid transporter (6-8), N-acetyleneuraminic acid lyase (57,58), NanK, N-acetylmannosamine-6-phosphate 2-epimerase (59,60), N-acetylglucosamine-6-phosphate deacetylase and glucosamine-6-phosphate deaminase (12). Substrates: N-acetyleneuraminic acid, N-acetylmannosamine, N-acetylmannosamine-6-phosphate, N-acetylglucosamine-6-phosphate, fructose-6-phosphate.

B) Structures of sugar-bound ROK and non-ROK sugar kinases have a highly-conserved sugar binding site. Glucokinase family *E. coli* glucokinase in pink (PDB: 1SZ2), ROK *S. griseus* glucokinase in green (PDB: 3VGL), ROK *B. subtilis* fructokinase in cyan (PDB: 3LM9) and ROK human bi-functional UDP-N-acetylglucosamine 2-epimerase/NanK in red (PDB: 2YHY).

C) This image shows the orientation of the ExGH motif (green) and ROK Zn-binding motif (red) relative to the sugar binding site (PDB: 3VGL).

**Figure 1.** A) A schematic of sugar flow through the bacterial sialic acid catabolism pathway. NanK catalyses the conversion of N-acetylmannosamine and ATP to form N-acetylmannosamine-6-phosphate and ADP. This proposed pathway is based on experimental evidence from *S. aureus* (4), *B. subtilis* and *E. coli* (55), as well data from KEGG genome data (56). For clarity, substrate and enzyme names are abbreviated. Enzymes: Sialic acid transporter (6-8), N-acetyleneuraminic acid lyase (57,58), NanK, N-acetylmannosamine-6-phosphate 2-epimerase (59,60), N-acetylglucosamine-6-phosphate deacetylase and glucosamine-6-phosphate deaminase (12). Substrates: N-acetyleneuraminic acid, N-acetylmannosamine, N-acetylmannosamine-6-phosphate, N-acetylglucosamine-6-phosphate, fructose-6-phosphate. B) Structures of sugar-bound ROK and non-ROK sugar kinases have a highly-conserved sugar binding site. Glucokinase family *E. coli* glucokinase in pink (PDB: 1SZ2), ROK *S. griseus* glucokinase in green (PDB: 3VGL), ROK *B. subtilis* fructokinase in cyan (PDB: 3LM9) and ROK human bi-functional UDP-N-acetylglucosamine 2-epimerase/NanK in red (PDB: 2YHY). C) This image shows the orientation of the ExGH motif (green) and ROK Zn-binding motif (red) relative to the sugar binding site (PDB: 3VGL).
Figure 2. Kinetic assays with N-acetylmannosamine kinase. A) Rate versus N-acetylmannosamine and ATP concentration fitted with the ternary complex kinetic model: $K_M$ (N-acetylmannosamine) = 0.30 ± 0.01 mM, $K_M$ (ATP) = 0.7 mM, $V_{max}$ = 497 ± 7 µmol/min/mg, $k_{cat}$ = 263 ± 3 s$^{-1}$. B) Rate versus N-acetylgulosamine concentration fitted to the Michaelis-Menten model: $K_{M_{app}}$ = 0.26 mM, $V_{max}$ = 114 µmol/min/mg, $k_{cat_{app}}$ = 23.4 ± 0.2 s$^{-1}$. C) Rate versus glucose concentration fitted to the Michaelis-Menten model: $K_M$ = 15 ± 1 mM, $V_{max}$ = 44.4 ± 0.3 µmol/min/mg, $k_{cat}$ = 60 ± 1 s$^{-1}$. 
Figure 3. Monomeric structure of SaNanK. A) The monomer has two domains; an N-terminal α/β domain (red) and a larger α+β domain (pink). The hinge region (green) joins the two domains and involves two α-helices shared between the domains. The active site is opposite the hinge region between the two domains. B) The active site residues of ROK kinases are highly conserved. Sequence alignment of ROK kinases listed in Table S1. Active sites residues are highly conserved (maroon) and residues elsewhere are poorly conserved (cyan).
Figure 4. Oligomeric state of SaNanK. A) Sedimentation velocity experiments with SaNanK at three concentrations (0.3, 0.6, 0.9 mg ml⁻¹; 9.5, 18.9, 27.4 µM). The fit of the model to the data (Fig. S4) provides a molecular mass of approximately 62 kDa, corresponding to a dimer (monomer mass is 31.7 kDa). B) Small-angle x-ray scattering of SaNanK (6 mg ml⁻¹). Ligand-free SaNanK experimental data (red circles) corresponds with theoretical scatter (red line), with a reduced $\chi^2$ of 1.7 vs. 4.9, compared to the N-acetylmannosamine crystal structure scatter (blue line). C) Structure of the SaNanK dimer, which is formed through residues of the larger $\alpha+\beta$ domain (pink), with the hinge region (green) and large N-terminal $\alpha/\beta$ domain (red) free in solution. D) The dimeric interface of SaNanK. The electrostatic surface (generated using PyMOL) shows three opposing negative and positive areas, which pair when the dimer interface forms. Residues involved with dimerisation were determined using PDBe PISA and were mapped onto the surface of SaNanK.
Figure 5. Conformational change in SaNanK. A) Comparison of ligand-free and N-acetylmannosamine-bound structures demonstrates that the N-terminal α/β domain (red to white) closes around N-acetylmannosamine, pivoting at the hinge region (green). B) Expected catalytic residues of SaNanK. The N-terminal α/β domain moves bringing Asp7 and Thr11 towards the active site, which in conjunction with Thr134 prime ATP for nucleophilic attack. The expected position of ATP was indicated by aligning the human NanK structure with N-acetylmannosamine and Mg.ADP bound (PDB: 2YHY). Asp108 binds and deprotonates the C6 hydroxyl of N-acetylmannosamine, increasing nucleophilicity of the sugar. C) Small-angle x-ray scattering of SaNanK (6 mg ml⁻¹) and N-acetylmannosamine (5 mM). Experimental data (blue circles) corresponds with theoretical scatter of the N-acetylmannosamine-bound crystal structure (blue line), with a reduced $\chi^2$ of 1.9 vs. 9.9, compared to the ligand-free crystal structure scatter (red line). D) Radius of gyration calculated from intensity during small-angle x-ray scattering. The radius of gyration was calculated to be condensed when N-acetylmannosamine is bound (26.9 Å vs 27.5 Å), which is consistent with the inward shift represented by the crystal structure.
Figure 6. Involvement of SaNanK Tyr160 in substrate association. A) N-acetylmannosamine-bound SaNanK. Gly68, Asn107, Asp108, Glu157 and Glu172 bind the hydroxyl groups of N-acetylmannosamine and Gly81, Glu157 and Tyr160 bind the C2 N-acetyl group. B) Comparison of sugar substrate binding in SaNanK (blue) S. griseus glucokinase (green) and Human NanK (pink). In SaNanK, the ExGH motif histidine is replaced by a tyrosine (Tyr160), which binds the larger N-acetylated substrate by tilting backwards from the active site and having greater length than histidine. In Human NanK, ExGH motif histidine bind the C1 hydroxyl and the axial N-acetyl group is bound by other non-conserved residues. C) N-acetylglucosamine-bound SaNanK. The N-acetyl carbonyl group is not able to hydrogen bond with Tyr160. D) Tyr160 provides substrate specificity towards N-acetylmannosamine, over N-acetylglucosamine. When tested with N-acetylmannosamine (blue, left axis), wild-type SaNanK has more substrate specificity (870 mM$^{-1}$ s$^{-1}$) than Tyr160Phe substituted enzyme (170 mM$^{-1}$ s$^{-1}$), which are absent the Tyr160 hydroxyl. When tested with N-acetylglucosamine (orange, right axis), wild-type SaNanK has less substrate specificity (90 mM$^{-1}$ s$^{-1}$) than Tyr160Phe substituted enzyme (390 mM$^{-1}$ s$^{-1}$), demonstrating that Tyr160 provides substrate specificity against N-acetylglucosamine. (Add A-F onto the panes as I have added in the text)
Figure 7. Arginine stacking feature in the region equivalent to the ROK Zn-binding motif. A) Comparison of region equivalent to the ROK Zn-binding motif (grey). In non-Zn-binding ligand-free SaNanK (red) the region is positioned away from the sugar active site. In Zn-binding ROK S. griseus glucokinase (PDB: 3VGK) (green) the region partially covers the sugar active site. Glucokinase family E. coli glucokinase (PDB: 1Q18) (yellow) has a larger region featuring an α-helix and is positioned away from the sugar active site. N-Acetylmannosamine and ATP substrates were aligned from N-acetylmannosamine-bound SaNanK and ROK kinase (PDB: 3VGL). B) In the region equivalent to the Zn-binding motif (blue), SaNanK features a pi-stacking arginine pair, which are coordinated by three negative residues. Two of the negative residues are part of a large α-helix (pink) stabilising the protein loop and positioning it away from the active site. Stable positioning of the region is important as it features three active site residues (Gluc157, Tyr160 and Glu172), which bind to N-acetylmannosamine (black). C) Differential scanning fluorimetry with SaNanK (solid line) and SaNanK-Arg164Lys (dashed line). SaNanK-Arg164Lys has lower thermal stability with the average melting temperature of the two domains is 4.1 °C lower than wild-type.
The basis for non-canonical ROK family function in the N-acetylmannosamine kinase from the pathogen Staphylococcus aureus

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