Identification of a Functionally Important Negatively Charged Residue Within the Second Catalytic Site of the SUR1 Nucleotide-Binding Domains

Jeff D. Campbell,1,2 Peter Proks,1 Jonathan D. Lippiat,1 Mark S.P. Sansom,2 and Frances M. Ashcroft1

The ATP-sensitive K⁺ channel (KATP channel) couples glucose metabolism to insulin secretion in pancreatic β-cells. It is comprised of sulfonylurea receptor (SUR)-1 and Kir6.2 proteins. Binding of Mg nucleotides to the nucleotide-binding domains (NBDs) of SUR1 stimulates channel opening and leads to membrane hyperpolarization and inhibition of insulin secretion. To elucidate the structural basis of this regulation, we constructed a molecular model of the NBDs of SUR1, based on the crystal structures of mammalian proteins that belong to the same family of ATP-binding cassette transporter proteins. This model is a dimer in which there are two nucleotide-binding sites, each of which contains residues from NBD1 as well as from NBD2. It makes the novel prediction that residue D860 in NBD1 helps coordinate Mg nucleotides at site 2. We tested this prediction experimentally and found that, unlike wild-type channels, channels containing the SUR1-D860A mutation were not activated by MgADP in either the presence or absence of MgATP. Our model should be useful for designing experiments aimed at elucidating the relationship between the structure and function of the KATP channel. Diabetes 53 (Suppl. 3):S123–S127, 2004

The ATP-sensitive K⁺ channel (KATP channel) plays a crucial role in insulin secretion by coupling β-cell metabolism to insulin secretion (1). At low glucose levels, the KATP channel is open, keeping the resting membrane potential hyperpolarized and preventing electrical activity and insulin secretion (2). Increased glucose metabolism results in closure of the KATP channels, leading to a depolarization of the β-cell membrane, initiation of electrical activity, opening of voltage-gated calcium channels, and an influx of Ca²⁺ ions that triggers the release of insulin granules.

Two very different protein subunits make up the KATP channel (3). The pore is composed of four inwardly rectifying K⁺ channel (Kir channel) subunits (Kir6.2), and each of these is associated with a sulfonylurea receptor (SUR) subunit (SUR1), which modulates the opening and closing (gating) of the channel. Metabolically generated changes in intracellular adenine nucleotides influence channel gating via interaction with both types of subunit. Thus, ATP (and ADP) closes the channel by binding to Kir6.2, whereas channel opening is mediated by interaction of Mg nucleotides (MgATP and MgADP) with SUR1 (4,5).

SUR belongs to the ATP-binding cassette (ABC) family of transporter proteins (6). Like other ABC transporters, it has two sets of transmembrane domains (TMDs), each containing six membrane spanning helices, and two nucleotide binding domains (NBDs). It also possesses an additional NH₂-terminal set of five transmembrane helices (Fig. 1A). The NBDs of all ABC proteins are highly conserved and contain several motifs involved in ATP binding and hydrolysis, including a Walker-A (WA) and Walker-B (WB) motif linked by a “signature” sequence (LLSGGQ) and two shorter sequences containing conserved glutamine (Gln-loop) and histidine (His-loop) residues (Fig. 1A). The crystal structures of a number of isolated NBDs suggest that their topology is also highly conserved across both prokaryotes and eukaryotes. Furthermore, the X-ray structures show that the NBDs form a dimer containing two nucleotide-binding pockets. These do not correspond to the NBDs encoded in the primary sequence, however, because the ATP molecules are sandwiched between the WA of one NBD and the signature sequence of the opposite NBD (Fig. 1B). For this reason, we henceforth refer to the two nucleotide-binding pockets as site 1 (Walker motifs of NBD1, linker NBD2) and site 2 (Walker motifs of NBD2, linker NBD1).

Because the sequences of NBD1 and NBD2 of SUR1 are not identical, the structure of these two sites will also not be identical. This may provide a structural explanation for the observed differences in function between the two sites of SUR1 (8). As described previously (7), it is possible to equate the properties described for NBD1 in functional studies with those of site 1 and to equate those described for NBD2 with site 2 (8). Therefore, site 1 binds nucleotides with higher affinity than site 2 (half-maximal inhibitory concentration [IC₅₀] of 4 and 60 μmol/l for ATP and 26 and 100 μmol/l for ADP at sites 1 and 2, respectively). In addition, ATP is preferentially hydrolyzed at site 2.

To examine these differences in nucleotide handling...
further, we built a molecular model of the SUR1-NBD heterodimer using a combination of mammalian and bacterial crystal structures as templates. We then mutated residues putatively identified in the model as being involved in nucleotide handling or catalysis and tested their functional effects on K\textsubscript{ATP} channel activity.

**RESEARCH DESIGN AND METHODS**

**Modeling.** The sequences of NBD1 and NBD2 of SUR1 were aligned with those of CFTR (cystic fibrosis transmembrane regulator) (protein database [PDB] code 1ROZ) and TAP1 (transporter associated with antigen processing) (PDB code 1JJ7), respectively, using ClustalW (9) and individual monomers generated using MODELLER 6 (version 2) (10). The individual NBD models were then least-squares fitted to the MJ0796 dimer crystal structure (PDB code 1L2T) (11). Molecular graphics diagrams were generated using VMD (visual molecular dynamics) (12) and PovRay (http://www.povray.org). Molecular dynamic simulations were performed as previously described (13).

**Electrophysiology.** *Xenopus* oocytes were defolliculated and coinjected with \(5\) ng each of mRNAs encoding Kir6.2 (GenBank accession no. D50581) and either wild-type or mutant SUR1 (GenBank accession no. L40624). The final injection volume was \(50\) nl per oocyte. Isolated oocytes were maintained as previously described (14), and currents were studied \(1-4\) days after injection. Macroscopic currents were recorded from giant excised inside-out patches at \(20-24^\circ\)C (14). The pipette solution contained (in mmol/l) 140 KCl, 1.2 MgCl\(_2\), 2.6 CaCl\(_2\), and 10 HEPES (pH 7.4 with KOH), and the internal (bath) solution contained 110 KCl, 1.44 MgCl\(_2\), 30 KOH, 10 EGTA, 10 HEPES (pH 7.2 with KOH), and nucleotides as indicated. Currents were recorded with an Axopatch 200B amplifier (Axon Instruments), filtered at 0.2 kHz and sampled at 0.5 kHz.

**RESULTS AND DISCUSSION**

**Modeling the SUR1 NBD heterodimer.** Because the sequence of NBD1 differs from that of NBD2, there are likely to be slight structural differences between the two NBDs. To take account of these differences, we used two different templates to construct a molecular model of the SUR1 heterodimer: NBD1 was modeled on the crystal structure of NBD1 of mouse CFTR (15) and NBD2 was modeled on the X-ray structure of the human TAP1 NBD (16). The two NBD models were then assembled in the nucleotide sandwich dimer configuration by least-squares fitting to the bacterial NBD homodimer MJ0796 (11). The CFTR-NBD1 and TAP1-NBD sequences share 33 and 26% sequence identity to the NBD1 and NBD2 of SUR1, respectively, which is the highest homology currently available for NBDs of known structure. Additionally, the CFTR-NBD1 and TAP1-NBD2 structures were cocrystallized with MgATP and MgADP, respectively. Thus, our model will represent a structure with ATP docked at NBD1 and ADP docked at NBD2. It is thought that this corresponds to the “active” conformation of SUR.

**Predictions of the molecular model.** Figure 1B shows the model of the NBD heterodimer of SUR1, with ATP docked at site 1 and ADP docked at site 2. Given that site 2 shows an enhanced ability to hydrolyze ATP (17), we searched for negatively charged residues proximal to the phosphate tail of ATP that could participate in coordination and hydrolysis of the nucleotide. Two negatively charged residues, D1505 and E1506 in the WB motif of NBD2, were observed to line site 2. Mutation of either of these residues abolishes the ability of MgADP to stimulate channel activity (5,18), and E1506 is of particular interest.
because its mutation to lysine (E1506K) leads to congenital hyperinsulinism in humans (18). Our model also identified a third negatively charged residue, D860, which lies ~5 Å from the γ-phosphate and could potentially interact with ATP (Fig. 1C). It is noteworthy that, in contrast to the other negatively charged residues, which line site 2, D860 is contributed by the opposite NBD. The equivalent residue in NBD2 is D1512, which lines site 1. To examine whether D860 and D1512 are of functional importance, we mutated them, individually, to alanine.

**Effects of the D860A mutation on Mg nucleotide activation.** Figure 2 shows that MgADP stimulates K<sub>ATP</sub> channel activity approximately twofold in the absence of other nucleotides, in accordance with previous results (14). In contrast, when D860 in NBD1 of SUR1 was mutated to alanine, MgADP blocked rather than stimulated channel activity. The extent of block (62 ± 5%; n = 5) is similar to that seen in the absence of Mg<sup>2+</sup> for wild-type channels (~60%) (14). This suggests that the mutation abolishes the stimulatory effect of MgADP on the channel and unmasks the inhibitory effect of ADP that is mediated via the inhibitory site on Kir6.2 (19).

Addition of 100 μmol/l MgATP produced a greater block of Kir6.2/SUR1-D860A channels than of wild-type channels: 95 ± 1% (n = 3) compared with 85 ± 5% (n = 3) (Fig. 2). This has also been observed for mutation of the WA lysine (20) or WB aspartate (5) in either NBD1 or NBD2. It can be attributed to loss of MgATP hydrolysis at the NBDs of SUR1, which, in the case of the wild-type channel, enhances channel activity and shifts the relationship between channel inhibition and ATP concentration to higher ATP levels.

MgADP was unable to stimulate the activity of channels carrying the D860A mutation in the presence of ATP, in contrast to wild-type channels (Fig. 2). The equivalent mutation in NBD2 of SUR1 (D1512A) did not result in functional channels.

**Role of D860.** Taken together, our results demonstrate a
critical role for D860 in nucleotide activation of the KATP channel. Because MgADP activation was abolished, the effect of this mutation cannot be attributed solely to loss of MgATP hydrolysis. Instead, it must prevent either binding of Mg nucleotides to site 2 of SUR1 or transduction to Kir6.2 of the conformational change consequent on nucleotide binding. Additional experiments are required to determine which of these possibilities is correct.

Although we cannot say whether D860 simply participates in nucleotide binding or also acts as a hydrolytic residue, the presence of D860 alone is not responsible for the functional differences between site 1 and site 2. This is because an equivalent residue (D1512) lines site 1. However, there are likely to be differences in the way that D860 and D1512 function at their respective binding sites. This is because a nonconserved arginine residue, R1379, which is NH2-terminal to the WA motif of site 2, lies directly adjacent to D860 (Fig. 3A), whereas no such positively charged residue exists in site 1. Mutation of the equivalent arginine residue in SUR2B (R1344) impaired ADP-induced channel activation as effectively as mutation of the WA lysine (21). This suggests both R1379 and D860 play an important functional role: potentially, R1379 has the ability to undergo alternating charge-charge interactions with ATP and D860.

We investigated this hypothesis by molecular dynamics simulations of our SUR1 NBD model. We ran four simulations: the wild-type model with 1) ATP docked at both NBDs, or 2) ATP docked at NBD1 and ADP docked at NBD2, and 3, 4) the equivalent simulations of the D860A mutant. The wild-type simulations demonstrate that R1379 preferentially interacts with ADP rather than ATP (Fig. 3B). Furthermore, the D860A simulations demonstrate that removal of the negatively charged aspartate disrupts the interaction of R1379 with nucleotide; in contrast to the wild-type simulations, R1379 more strongly interacts with the ATP than with ADP. The reduction in the predicted interaction of R1379 with ADP following mutation of D860 is consistent with the impaired ability of MgADP to stimulate channel activity (Fig. 2).

CONCLUSION
We have demonstrated that an approach that combines molecular modeling predictions, simulations, and functional analysis is useful for understanding the structural biology of the NBDs of SUR1. Our results define a new residue, D860, which contributes to the complex mechanism by which nucleotide binding to these NBDs results in opening of the KATP channel.

ACKNOWLEDGMENTS
We thank the Wellcome Trust and the Royal Society for support. J.D.C. is a Wellcome Trust Structural Biology D.Phil student and a Linacre College Canadian National scholar, and F.M.A. is the GlaxoSmithKline Research Professor of the Royal Society.

REFERENCES


