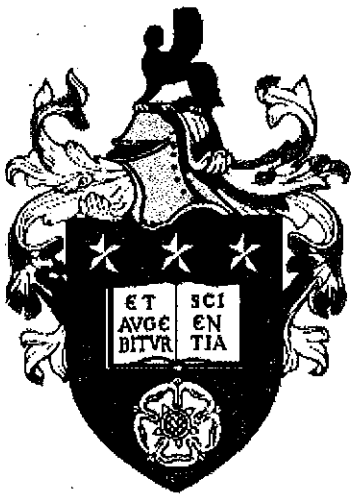
STRUCTURAL AND FUNCTIONAL STUDIES ON MEMBRANE TRANSPORT PROTEINS



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to be in the membrane when expressed in *E. coli* and only two proteins were expressed at levels <20% of the cysteine-less parent protein. Most of the mutants showed significant glutamate transport activity. Although mutation of E270, S316, D386 and N397 to cysteine inactivated transport, mutation to chemically more similar residues typically restored activity, at least in part. However, mutation of S316 to threonine, D390 to glutamate or N397 to glutamine failed to result in such restoration. In conjunction with molecular docking investigations and previous findings on glutamate transport proteins, the present study suggested that (a) D390, (b) T394 and N397 and (c) L313, S316 and R393C, lie in proximity to or make key interactions with the α-amino, α-carboxylate and γ-carboxylate groups of glutamate respectively. Moreover, evidence was obtained that S310, G356 and G359 are located on the permeant translocation pathway and are accessible from the periplasmic side of the membrane. Analysis by solid state NMR of mutants in which N397 had been replaced by cysteine or glutamine revealed that these proteins lacked transport activity because they had lost the ability to bind the permeant. FT-IR and CD spectroscopic analyses showed that this was not due to any major distortion in the proteins's secondary structure. In addition it proved possible to detect substrate-induced conformational changes in the protein following the labelling of mutant L376C with Alexa-546-maleimide. It is concluded that GltP offers an excellent model for probing molecular mechanisms in both bacterial and mammalian glutamate/aspartate transporters.

Finally, a novel approach to understanding the mechanisms of metal-ion transport proteins was developed using cadmium-113 as a solid state NMR probe of the *E. coli* zinc export protein, ZitB. Using this approach, the binding affinities (IC_{50}) of Cd^{2+}, Zn^{2+}, Cu^{2+} and Ni^{2+} to ZitB were found to be 15 ± 1, 80 ± 1.2, 12 ± 1 and 41 ± 2.5 mM respectively. In addition, this method was also applied for the analysis of several mutational variants of ZitB. It is anticipated that this methodology will be of wide applicability for investigating the ion selectivities and structure/function relationships of metal transport proteins in general.

Abstract

Membrane transport proteins are of central importance to living cells and have been implicated in a large number of diseases. As a first step towards understanding the molecular mechanisms by which these proteins transport nutrients, neurotransmitters, metal-ions and other substances across biological membranes, a systematic approach for their expression and affinity-tagging was devised which takes into account transmembrane topology. Using a set of bacterial transporters (NupG, ZitB, HI0736, GltP, UhpT, MntH, PitA, PutP and YchM) from nine distinct families with known and differing topologies, the efficacy was tested of a panel of conventional and Gateway™ recombinational cloning vectors. These were designed for protein expression under the control of the *tac* promoter and for the addition of differing N- and C-terminal affinity tags. For transporters in which both termini were cytoplasmic, C-terminal oligohistidine tagging by recombinational cloning typically yielded functional protein at levels equivalent to or greater than those achieved by conventional cloning. In contrast, recombinational cloning was not effective for examples of the substantial minority of membrane proteins that have one or both termini located on the periplasmic side of the membrane, possibly because of impairment of membrane insertion by the tag and/or *att*-site-encoded sequences. However, fusion either of an oligohistidine tag to cytoplasmic (but not periplasmic) termini, or of a *Strep*-tag II peptide to periplasmic termini using conventional cloning vectors did not interfere with membrane insertion, enabling high-level expression of such proteins. In conjunction with use of a C-terminal Lumio™ fluorescence tag, which was found to be compatible with both periplasmic and cytoplasmic locations, these findings offer a system for strategic planning of construct design for high throughput expression of membrane proteins for structural genomics projects.

As an example of the utility of being able to express membrane proteins at high levels, the structure/function relationships of the glutamate transporter GltP from *Escherichia coli* were investigated. In mammals, homologous glutamate transporters play important roles in neurotransmission and provide glutamate for the synthesis of γ-amino-butyric acid, glutathione and protein. Guided by a homology model and sequence alignments, 42 single-amino acid substitution mutants were generated in a cysteine-less form of GltP in order to probe the mechanism of transport. All the mutant proteins were found