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Review

Structure-based interpretation of the mutagenesis database for the nucleotide binding domains of P-glycoprotein

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Abstract

P-glycoprotein (P-gp) is the most intensively studied eukaryotic ATP binding cassette (ABC) transporter, due to its involvement in the multidrug resistance phenotype of a number of cancers. In common with most ABC transporters, P-gp is comprised of two transmembrane domains (TMDs) and two nucleotide binding domains (NBD), the latter coupling ATP hydrolysis with substrate transport (efflux in the case of P-gp). Biochemical investigations over the past twenty years have attempted to unlock mechanistic aspects of P-glycoprotein through scanning and site-directed mutagenesis of both the TMDs and the NBDs. Contemporaneously, crystallographers have elucidated the atomic structure of numerous ABC transporter NBDs, as well as the intact structure (i.e. NBDs and TMDs) of a distantly related ABC-exporter Sav1866. Significantly, the structure of P-gp remains unknown, and only low resolution electron microscopy data exists. Within the current manuscript we employ crystallographic data for homologous proteins, and a molecular model for P-gp, to perform a structural interpretation of the existing "mutagenesis database" for P-gp NBDs. Consequently, this will enable testable predictions to be made that will result in further in-roads into our understanding of this clinically important drug pump.

Keywords: ABC transporter; P-glycoprotein; Mutagenesis; Nucleotide binding domain; Mechanism; Catalytic cycle; Modeling

Contents

1. Introduction

The spectre of multidrug resistance continues to haunt the treatment of cancer, as well as bacterial, viral and parasitic infections [1]. One of the mechanisms contributing to cancer

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multidrug resistance is the over-expression of members of the ATP binding cassette (ABC) transporter family [1]. These proteins are envisaged as a front-line mechanism contributing to anti-cancer drug resistance, by limiting the intracellular accumulation of cytotoxic drug and hence reducing the efficacy. At least three human ABC transporters, namely P-glycoprotein (P-gp; ABCB1 [2]), multidrug resistance associated protein 1 (MRP1; ABCC1 [3]) and breast cancer resistance protein (BCRP; ABCG2 [4]), have been shown *in vitro* to have the capability to efflux a structurally and mechanistically diverse range of anti-cancer agents [5–7]. Additionally, the clinical impact of over-expressing these three ABC transporters on the prognosis of several tumour types has been documented [8].

ABC transporters are multidomain proteins and these domains may be encoded by different genes. However, a common minimum functional unit (at least for eukaryotic export ABC transporters) seems to consist of two transmembrane domains (TMDs), each of which contains multiple α-helices [9], and two cytoplasmic nucleotide binding domains (NBDs) [10]. The TMDs form substrate binding sites and provide a translocation path across the membrane [11–13], whilst the NBDs bind and hydrolyse ATP, the energy of which powers the drug export process [14]. A panoply of experimental data for P-gp and related ABC transporters supports the view that conformational changes are transmitted between these four domains and that the regulation of this allostery is crucial for correct overall protein function [15–17].

The most intensively investigated of this triumvirate of drug pumps is P-gp, which has now been studied for over three decades [18]. A subset of these investigations has been aimed at unlocking the molecular mechanism of P-gp, that is, to determine which amino acids are central in the recognition and transport of drugs and those which are critical for ATP binding and hydrolysis to name two examples. Moreover, studies aimed at determining how the two TMDs and the two NBDs communicate with each other have been performed in order to increase our understanding of conformational change transmission [14]. The majority of these studies have relied on site-directed or scanning mutagenesis to produce mutant isoforms of the protein, which are then tested for *inter alia* drug binding, drug transport, and ATPase activity (for examples, see [19–23]). In the latter case activity is measured both in the absence of drug, the so-called basal ATPase, and in the presence of drug, the so-called drug stimulated ATPase.

In parallel with these investigations, there have been advances in our understanding of the structure of ABC proteins [24–32]. Notably the major advances have been: i) the description at atomic resolution of the structure of an isolated NBD [26], ii) the prediction [33] and then the structural confirmation [29,34] of the correct interaction between a pair of NBDs [10] and iii) the first determinations of the complete structures of entire ABC transporters [25,27,35–37]. These exciting structural developments allow an interpretation of the massive wealth of mutagenesis data that has appeared during the past twenty years for P-gp. In the current review article, we focus on the NBDs of P-gp and revisit the published mutagenesis data on these. A broader account of the mutational analysis of ABC transporters has been provided recently [38]. We will illustrate how the interpretation of the effects of mutations on the NBDs can in some instances be revised based upon the structural data, and we provide an on-line resource which will grow in parallel with future experimentation. Some candidate residues for mutation are outlined in the final paragraphs.

2. The structure of the nucleotide binding domains and their association into an ATP-dependent dimer

Within ABC transporter NBDs there are present several sequence motifs (or even individual residues) which have a higher degree of conservation than the rest of the domain [39]. At least 8 such sequences have been described (Table 1). Of these, the Walker-A (GxxGxGK{S/T}, where x is any residue) and Walker-B (θθθθDE, where θ is any hydrophobic residue; note that for convenience we include the highly conserved glutamate C-terminal to the aspartate in this consensus) are present in many other classes of ATPases and are known to make non-covalent interactions with the triphosphate moiety of ATP, and to catalyse the hydrolysis of the γ-phosphate [40]. The other motifs are specific to ABC transporters, and several other families of proteins which employ the ABC transporter NBD as an energising module for other functional systems such as DNA repair and translational control [34,41–44]. Of these specific motifs the signature sequence, D-loop, X-loop and the His-loop can be defined by conserved motifs (LSGGQ, SALD, xVG{D/E}{K/R}G, and βθβμH respectively, where β is a branched aliphatic residue, and μ is a hydrophilic residue), whereas the Gln-loop and the A-loop are identified as having a highly conserved residue (any aromatic in the case of the A-loop) approximately 30 amino acids C-terminal and 20 amino acids N-terminal to the Walker-A motif respectively. The functions and importance of these sequences have been intensively researched, and a structural corroboration has been possible in some cases [39]. However, we are still considerably short of

Table 1
Conserved ABC transporter motifs in P-glycoprotein

Motif name	Sequence	P-gp residue		Proposed function
		N-half	C-half	
A-loop	FxYPX	401	1044	Aromatic ring stacking with adenosine
Walker-A	GxSGCGKS	427–434	1070–1077	ATP binding pocket formation
Gln-loop	VSQEP	473–477	1116–1120	Inter-domain communication role
X-loop	TxVG{E/D}{R/K}G	522–528	867–873	Inter-domain communication role
Signature	LSGGQ	531–536	1176–1181	Cross-dimer ATP binding pocket formation
Walker-B	ILLLDE	551–556	1196–1201	ATP binding pocket formation
D-loop	SALD	559–562	1204–1207	Cross-dimer interaction
His-loop	IVIAH	583–587	1228–1232	ATP-dependent switch region

For each motif we give the sequence location of the motif in each half of human P-gp, the flanking sequence (x represents any amino acid) and the function of each.

being able to understand how each of these elements in the NBDs coordinates functionally with the other motifs and contributes to the overall mechanism of P-gp.

The first glimpses into the structures of ABC transporters came with the 1.5 Å structure of the NBD of the histidine importer from *S. typhimurium*, HisP [26]. Since then, at least 20 NBD structures have been published [39] and there are several dozens more deposited in the Protein Data Bank. The structure of the single NBD is essentially composed of two sub-domains [9,10,39]; firstly, an α/β sub-domain which shows some structural homology to the F1-ATPase and RecA structures and contains four of the conserved motifs (Walker-A, Walker-B, His-loop and A-loop; discussed below) present in all ABC transporter NBDs. Secondly, a primarily α-helical sub-domain exists, encompassing the signature motif, and including a region with greater diversity between NBDs of ABC transporters of unrelated function [39]. The exact orientation of the two sub-domains (which is approximated by an L-shape) is influenced by the nucleotide occupancy of the domain [39], and two further conserved motifs (the D-loop and Gln-loop) are located at the sub-domain interface.

The crystallization of NBDs from various ABC transporters in a number of different dimeric associations led to an initial confusion over the precise dimeric interaction of two NBDs [10]. The use of selected mutant isoforms of several NBDs has allowed this dimer interface to be unequivocally determined now. The dimer is frequently likened to a sandwich, with nucleotide being the filling between two NBDs. For example, the NBD of rat TAP1 crystallized as a sandwich dimer when the Walker-B C-terminal acidic aspartate was neutralized to asparagine [28], the equivalent mutation in MJ0796 produced the same structural apposition [45] and an alternative mutation (substitution of the conserved histidine of the His-loop with alanine) produced the same conformation in the NBD of the haemolysin transporter [32]. The apposition of nucleotide at the NBD:NBD interface generates composite ATP binding sites involving residues in the Walker-A and -B motifs of one NBD, and the signature motif of the other NBD [29,43,46].

3. Structure of P-gp

Whilst there has been an explosion in the structural determination of NBDs, progress with P-gp has been at lower resolution. In particular the electron microscopic studies of Ford et al. have gradually improved in resolution from an initial 25 Å to 8–10 Å (although with loss of resolution in the plane perpendicular to the membrane [15,47–49]). Even at this resolution it remains difficult to map the constituent domains onto the electron density with any certainty. This has necessitated the building of structural homology models in order to underpin biochemical and pharmacological investigations into the pump. However, these have been primarily based upon three structures for MsbA, all of which have subsequently been retracted due to errors in crystallographic data miscalculation [50]. In light of the need to recalculate the MsbA structures [50] it is worth explaining that features of the NBD:NBD interface may be correctly represented in these homology models (if built based upon the structure of the *S. typhimurium* MsbA in the presence of ADP and vanadate [51]). However, the details of the NBD:TMD and TMD α-helical packing are likely to be incorrect [52–54]. Thus, the advent of the structure of Sav1866 [25], a bacterial putative multidrug exporter has enabled more realistic homology models of P-gp to be constructed [55]. As a consequence we are able to now consider the mutagenesis database for P-gp and correlate it with our structural and functional understanding of this protein.

4. Approaches used in the site-directed and site-scanning mutagenesis of P-gp NBDs

The extensive mutagenesis of the NBDs of P-gp does not always have predictable outcomes. Whilst mutations in certain of the most conserved regions may have a predictable effect, there remain many mutations which can only be interpreted in the light of structural data, and other mutations which can be reinterpreted in the light of newer structural data. Before discussing these it is worth briefly diverting to consider the types of experimental approach that have been used by several research groups to probe the function of mutant isoforms of P-gp.

Within this review we have included analyses of mutations that are made in one of three backgrounds. Two of these backgrounds can be considered wild type (human MDR1 P-gp and mouse MDR3 P-gp). The third is a mutant background in which the seven endogenous cysteines of P-gp have been replaced by alanine [56] or serine [57]. In each case the cysteine-less isoform has been characterized and shown to possess normal basal and drug stimulated ATPase activity, to be fully functional for transport [56,57], and to have a pharmacological profile essentially indistinguishable from the wild type P-gp [57].

The characterization of mutations again broadly falls into two distinct classes; those in which the protein was purified and those in which it was not. In the latter case, typical experiments to characterize the broad effect of mutation on protein function are whole cell transport assays (which may be radioactivity or fluorescence based), cell cytotoxicity experiments, functional complementation, or membrane based assays such as cross-linking and residue accessibility studies. Experimental studies of purified protein (and in some instances reconstituted protein) are by their nature able to discern more precisely the effect of residue substitution. Reconstitution of the protein allows the ATPase activity of the protein in both the basal state (no drug substrate) and the drug stimulated state to be investigated, thus reporting on the coupling of the NBDs with the events at the TMDs. Additionally, the specific nucleotide occupancy may be investigated as an experimental variable by examination of P-gp in multiple conformational states including the resting -state (no nucleotide bound), the ATP bound state (in the presence of AMP-PNP, but see [39,58] for a discussion of caveats) and the post-hydrolytic state (ADP.vanadate trapped conformation). The use of radioactive, photoactive derivatives of ATP ([^{32}P]-labelled azido-ATP) has proved valuable in these endeavours.

Within the following discussion we will try to include salient experimental details to enable comparison of similar functional assays on mutant isoforms of the protein. In the ensuing paragraphs we concern ourselves only with mutations made in

full length P-gp, and have excluded any mutations made within the isolated NBDs.

5. Structural templates for interpretation of P-gp NBD mutational analysis

Our structural (re)-interpretations of mutagenesis data involve mapping residues onto two templates (Fig. 1). The first is that for the bacterial exporter Sav1866, from *S. aureus*, which has been solved to a resolution of 3.0 Å in the presence of ADP, although the structure is believed to be equivalent to an outward facing ATP bound state [25]. Sav1866 crystallizes as a homodimer with the two NBDs adopting a sandwich dimer configuration. The important aspect of this structure for the current interpretation is the "domain swapping" observed, i.e. that the TMD of one Sav1866 monomer makes extensive contacts through two intracellular linker regions (ICLs) to the NBD of the opposite Sav1866 monomer [25]. Rather than use the structure of Sav1866 itself we have used a model of P-gp based upon the Sav1866 structure (Fig. 1a). The model is described in full by O'Mara and Tieleman [55]. The second structural data we employ is the high resolution structure of a rat TAP1 NBD homodimer [28], in which each TAP1 bears an Asp→Asn mutation at position 645, which is comparable to the

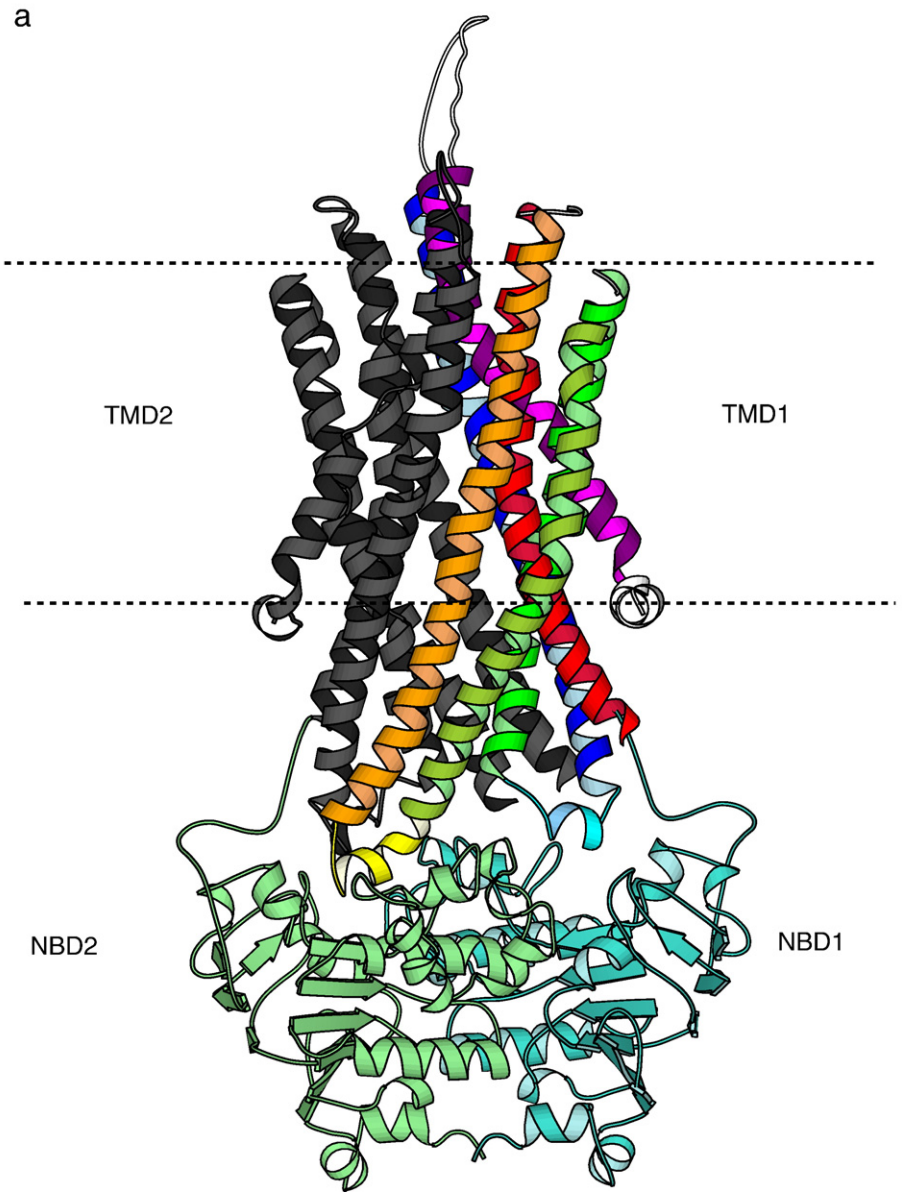

Fig. 1. Structural templates used to infer the role of residues within P-gp. a. Homology model of human P-gp based upon the structure of Sav1866 [25]. The model is rendered in a ribbon representation with the NBDs in cyan (NBD1) and light green (NBD2) respectively. The approximate location of the membrane is indicated by dotted lines. The TM helices and intracellular connecting loops of TMD1 are coloured as follows: TM1 magenta, TM2 blue, ICL1 cyan, TM3 bright green, TM4 yellow-green, ICL2 yellow, TM5 orange, TM6 red. b. Structure of the rat TAP NBD1 homodimer [28]. The two NBDs are in different degrees of grey shading, with the conserved sequence motifs highlighted as follows: aromatic-loop red; Walker-A orange; Gln-loop yellow; X-loop dull green; signature bright green; Walker-B cyan; Asp-loop blue; His-loop magenta.

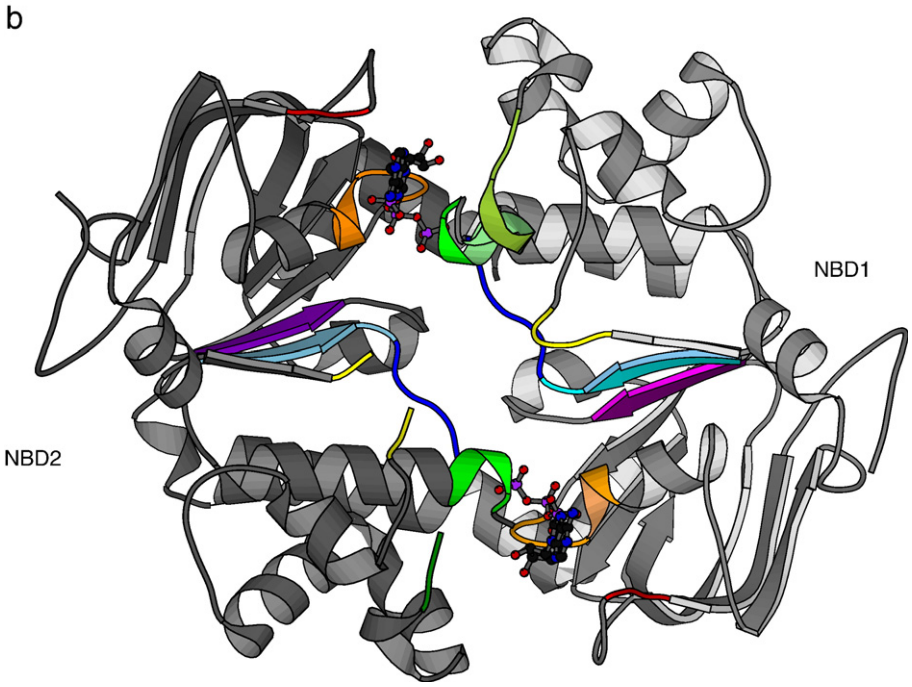

Fig. 1 (*continued*).

glutamic acid residue at the extreme C-terminus of the Walker-B motif (residues 556 and 1201 in P-gp). This structure was solved to 2.0 Å resolution with two ATP molecules coordinated in the sandwich dimer arrangement [28], and is employed as an interpretative tool as it is the highest resolution NBD dimer structure, is from an eukaryotic ABC transporter, and shares 45% sequence identity in the NBDs with P-gp (Fig. 1b). A multiple sequence alignment of the NBDs of Sav1866, human and mouse P-gp (N- and C-terminal sequences) and rat TAP1 was constructed with ClustalW [59], and was used without manual refinement to determine equivalent residues for structural mapping purposes (Fig. 2). These 6 sequences share between 36% (TAP1:Sav1866) and 92% (human:mouse NBD1) sequence identity. All structural mapping was done using RasTop as a visualization aid, with further molecular structure diagrams generated using MolScript [60].

For each mutant isoform we have prepared an entry in a table (Supplementary Tables S1–S4) which defines the residue mutated, indicates its conservation, the functional effect, the original interpretation, relevant structural details obtained from mapping onto TAP1 and a Sav1866-based P-gp model, and a revised structural interpretation where appropriate. Several of these are discussed in more detail in the following sub-sections. The Supplementary Table and accompanying text is split into 3 comparable sub-tables, discussing mutations involved in ATP binding/hydrolysis (Table S1), mutations at the NBD:NBD interface (Table S2), and mutations at the NBD:TMD interface (Table S3). This partitioning of the data is purely for convenience as of course residues at the NBD:NBD interface may additionally affect ATP binding and hydrolysis. A fourth table (Table S4) consisting of mutations which either do not fit conveniently into one of the other three tables, or which impart relatively little mechanistic knowledge is also appended. All four tables will be continuously updated at our website, http://www.nottingham.ac.uk/~mbzidk/P-gp%20Mutations.htm. Throughout the text we use both the single letter and three letter amino acid codes, but refer to mutations using the format X123Z to denote replacement of residue X at position 123 with residue Z.

6. Mutations involved in conserved motifs critical for ATP binding and hydrolysis (Table S1)

The majority of mutations that fall under this banner have either predictable effects on ATP binding and/or hydrolysis, or have been made in the light of early structural data (i.e. subsequent to the publication of a monomeric NBD [26]) which is substantiated by more recent data. For example, the recent mutation of the conserved aromatic amino acid of the A-loop produced data which was consistent with an absolute requirement for an aromatic residue (Phe or Tyr) at A-loop positions in NBD1 and NBD2 for full ATPase activity and transport activity [61,62]. Even substitution of the existing tyrosines with tryptophans was only partially tolerated (a double Tyr→Trp mutant was non-functional in transport assays) [61]. Although structural data (Fig. 3) clearly demonstrates that the aromatic ring stacks in a π–π fashion with the adenine ring of bound nucleotide [25,26,28], none of the investigations at this position (within P-gp) has so far been able to demonstrate why ATP is the preferred nucleotide substrate for P-gp. Although the individual NBDs of P-gp can bind CTP and GTP [58], and membrane vesicles isolated from P-gp over-expressing cells can perform GTP-dependent drug transport at a rate approximately 75% that supported by ATP [63], the purified protein displays only 7–12% activity when incubated with GTP, compared to

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MDR1_N-half          371  NKPSIDSYSKSGHKPDNIK-GNLEFRNVHFSYPSRKEVKILKGLNLKVQSGQTVALVGNS
Mouse_MDR3_NBD1      367  NKPSIDSFSKSGHKPDNIQ-GNLEFKNIHFSYPSRKEVQILKGLNLKVKSGQTVALVGNS
MDR1_C-half          1014 KTPLIDSYSTEGLMPNTLE-GNVTFGEVVFNYPTRPDIPVLQGLSLEVKKGQTLALVGSS
Mouse_MDR3_NBD2      1010 KTPEIDSYSTQGLKPNMLE-GNVQFSGFVFNYPTRPSIPVLQGLSLEVKKGQTLALVGSS
Sav1866              318I  DEDYDIKNGVGAQPIEIKQGRIDIDHVSFQYNDN-EAPILKDINLSIEKGETVAFVGMS
RAT_TAP1_NBD         470  -----------SLAPLNMK-GLVKFQDVSFAYPNHPNVQVLQGLTFTLYPGKVTSLVGPN

MDR1_N-half          430  GCGKSTTVQLMQRLYDPTEGMVSVDGQDIRTINVRFLREIIGVVSQEPVLFATTIAENIR
Mouse_MDR3_NBD1      426  GCGKSTTVQLMQRLYDPLDGMVSIDGQDIRTINVRYLREIIGVVSQEPVLFATTIAENIR
MDR1_C-half          1073 GCGKSTVVQLLERFYDPLAGKVLLDGKEIKRLNVQWLRAHLGIVSQEPILFDCSIAENIA
Mouse_MDR3_NBD2      1069 GCGKSTVVQLLERFYDPMAGSVFLDGKEIKQLNVQWLRAQLGIVSQEPILFDCSIAENIA
Sav1866              377  GGGKSTLINLIPRFYDVTSGQILIDGHNIKDFLTGSLRNQIGLVQQDNILFSDTVKENIL
RAT_TAP1_NBD         518  GSGKSTVAALLQNLYQPTGGKVLLDGEPLVQYDHHYLHTQVAAVGQEPLLFGRSFRENIA

MDR1_N-half          490  YGREN--VTMDEIEKAVKEANAYDFIMKLPHKFDTLVGERGAQLSGGQKQRIAIARALVR
Mouse_MDR3_NBD1      486  YGRED--VTMDEIEKAVKEANAYDFIMKLPHQFDTLVGERGAQLSGGQKQRIAIARALVR
MDR1_C-half          1133 YGDNSRVVSQEEIVRAAKEANIHAFIESLPNKYSTKVGDKGTQLSGGQKQRIAIARALVR
Mouse_MDR3_NBD2      1129 YGDNSRVVSYEEIVRAAKEANIHQFIDSLPDKYNTRVGDKGTQLSGGQKQRIAIARALVR
Sav1866              437  LGRPT--ATDEEVVEAAKMANAHDFIMNLPQGYDTEVGERGVKLSGGQKQRLSIARIFLN
RAT_TAP1_NBD         578  YGLTR-TPTMEEITAVAMESGAHDFISGFPQGYDTEVGETGNQLSGGQRQAVALARALIR

MDR1_N-half          548  NPKILLLDEATSALDTESEAVVQVALDKAR--KGRTTIVIAHRLSTVRNADVIAGFDDGV
Mouse_MDR3_NBD1      544  NPKILLLDEATSALDTESEAVVQAALDKAR--EGRTTIVIAHRLSTVRNADVIAGFDGGV
MDR1_C-half          1193 QPHILLLDEATSALDTESEKVVQEALDKAR--EGRTCIVIAHRLSTIQNADLIVVFQNGR
Mouse_MDR3_NBD2      1189 QPHILLLDEATSALDTESEKVVQEALDKAR--EGRTCIVIAHRLSTIQNADLIVVIQNGK
Sav1866              495  NPPILILDEATSALDLESESIIQEALDVLS--KDRTTLIVAHRLSTITHADKIVVIENGH
RAT_TAP1_NBD         637  KPRLLILDDATSALDAGNQLRVQRLLYESPEWASRTVLLITQQLSLAERAHHILFLKEGS

MDR1_N-half          606  IVEKGNHDELMKEKGIYFKLVTMQTAGNEVELENA----
Mouse_MDR3_NBD1      602  IVEQGNHDELMREKGIYFKLVMTQTAGNEIELGNEACKS
MDR1_C-half          1251 VKEHGTHQQLLAQKGIYFSMVSVQAGTKRQ---------
Mouse_MDR3_NBD2      1247 VKEHGTHQQLLAQKGIYFSMVSVQAGAKRS---------
Sav1866              553  IVETGTHRELIAKQGAYEHLYSIQNL-------------
RAT_TAP1_NBD         697  VCEQGTHLQLMERGGCYRSMVEALAAPSD----------
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Fig. 2. Sequence alignment of the NBDs of human and mouse P-gp, Sav1866 and rat TAP1. Sequences were aligned by ClustalW and the multiple alignment was not manually adjusted. Consensus motifs are highlighted as coloured in Fig. 1b.

ATP [64,65]. Related mutagenic studies on the NBD of an *E. coli* colicin transporter, CvaB, have shown that the amino acids 2 and 3 residues downstream of the conserved aromatic are determinants of nucleotide specificity [66]. Mutagenesis of equivalent residues in the P-gp aromatic loops may be required to fully understand nucleotide specificity, although there is no strong residue conservation with CvaB to guide mutant selection.

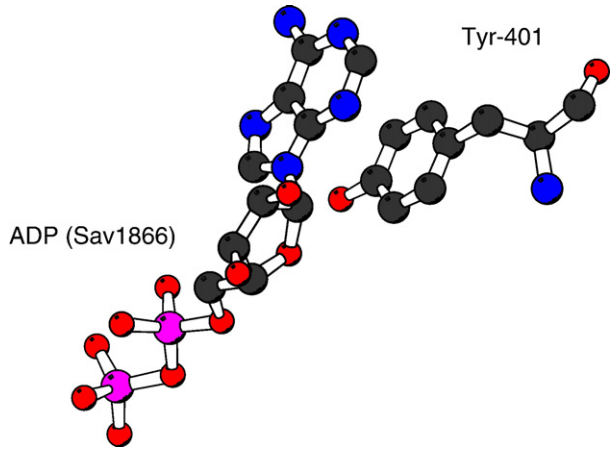

Fig. 3. Aromatic residue:nucleotide interactions in P-gp. The conserved aromatic residue upstream of the Walker-A motif in the NBD forms a stacking π−π interaction with bound nucleotide.

Residues within the Walker-A motif have also been the subject of intense mutagenesis activity, in particular the lysine residues 433 and 1076. A general consensus to emerge from these studies is that the Walker-A lysine is essential for ATP hydrolysis, with even a mutation of this lysine at one of the two NBDs preventing a single cycle of ATP hydrolysis in the presence of vanadate (evidenced by the ability to trap ADP. vanadate), whilst ATP binding was perturbed but not fully prevented — depending to some extent on the substituted residue [67–72]. As with the A-loop mutations these data can be explained by the structural data on the isolated NBD, without the requirement for detailed structural data on P-gp *per se*.

Two of the seven endogenous cysteine residues in human P-gp (Cys 431, Cys 1074) have proved useful in mutational analysis and cross-linking studies as they are positioned within the Walker-A motif (Table 1). Two studies have proposed that the Walker-A cysteine residues can be cross-linked to the opposite Walker-A motif [73,74], either through a direct disulphide between the two naturally occurring Walker-A cysteines [74] or between the Walker-A cysteine of NBD2 and a G427C Walker-A NBD1 mutation [73]. Examination of our structural templates, which are both in a nucleotide bound configuration, suggests that such a direct interaction is unlikely as the Cα atoms of these residues are in excess of 35 Å apart (Table S1). More recently, in the light of the NBD sandwich dimer, Loo et al. have demonstrated that the Walker-A cysteine can be cross-linked to a cysteine residue introduced within the opposite signature motif (L1176C, [19]), 10 Å apart (Cα–Cα, Table S1).

The distinction between these two, apparently irreconcilable findings may lie in the temperature required to effect oxidative cross-linking. Walker-A cross-linking to the signature motif could be effected at 4 °C, whereas the Walker-A:Walker-A cross-linking required 37 °C [19,73,74]. For the Walker-A motifs to be sufficiently close to each other during the catalytic cycle (which of course occurs at 37 °C) would still require a dramatic change in the NBD:NBD orientation. It is also worthy of mention here, that in addition to being involved in cross-linking studies, it has been firmly demonstrated that these Walker-A cysteine residues are the site of interaction with a number of sulphydryl reactive, non-specific inhibitors including *N*-ethylmaleimide (NEM), 4-chloro-7-nitro-1,2,3-benzoxadiazole (NBD-Cl), and 2-(4-maleimidylanilino)-naphthalene-6-sulfonic acid (MIANS), and that these reagents are able to inhibit P-gp ATPase in an ATP-protected manner [58,71,74–77]. Of these, MIANS has a linear length of at least 16 Å [78], suggesting that during the catalytic cycle there is a significant opening of the NBD dimer. Inhibition studies with larger maleimides may shed, complementary, further information on this separation (see Fig. 4).

Finally, within this section we consider the mutations made to the conserved Walker-B acidic amino acids Glu 556 in P-gp NBD1 and Glu 1201 in NBD2. This residue is often referred to as the "catalytic carboxylate", although Schmitt et al. distinguish between this residue as a general base and the possibility of substrate-assisted catalysis [32]. Neutralization of the acidic residue has, in studies on distinct ABC transporters, resulted in the detection of nucleotide bound tightly ("occluded") by the mutant protein [22,23,28,45,79]. Discounting the experimental trapping of ADP with vanadate, this tight binding of nucleotide (reported Kd for ATP is 9 μM in the occluded state [22]), is not seen in un-mutated P-gp (Kd for ATP is 0.5 mM [80]), and has prompted detailed investigations to focus on these residues. Single mutations (E552Q or E1197Q) in mouse MDR3 produced isoforms that were inactive in a functional complementation assay (export of FK506 from *S. cerevisiae*) [81]. Additionally, the purified mutant isoforms displayed undetectable basal and drug stimulated ATPase activities [81]. However, they retained the ability to trap ADP.vanadate following incubation with Mg.ATP, indicating that the hydrolysis of a single ATP molecule was still occurring, but that cycles of ATP hydrolysis were being impaired, potentially due to abrogated release of Mg.ADP [81]. A slightly different interpretation resulted from studies of double mutants in purified mouse MDR3 containing E→Q, E→A, E→D and E→K mutations at the equivalent Walker-B position [22] and from studies of human P-gp with a double E→Q mutation [82]. In these studies the double mutants showed a very low, but P-gp specific, ATPase activity (up to 1000-fold lower than wild type), which differed depending on the mutation introduced (e.g. E→A mutant was 2.5-fold more active than an E→K mutation) [22]. Loss of even a single methyl group from the Glu sidechain rendered P-gp several hundred fold less active than wild type demonstrating both that length of the sidechain and its chemistry is essential [22]. Detailed analysis of ATP binding in the presence of vanadate, and analysis of the bound species by thin layer chromatography provided an explanation for the effects of Glu mutations on P-gp [22,82]. Rather than being the result of impaired second cycle ATP hydrolysis, it was concluded that transition state formation was altered in the mutants, presumably due to the failure to co-ordinate nucleotide correctly, the mutants being able to stably trap ATP in a vanadate independent manner with a 1:1 molar stoichiometry [22,82]. An important question pertaining to these mutants is whether the occlusion of ATP represents a genuine step in the catalytic cycle of P-gp, or whether it is a side-effect of the mutations. Recent investigations support the occluded state being a true step in the catalytic cycle (Fig. 4 schematic). Firstly, residues which co-ordinate Mg.ATP prior to hydrolysis (e.g. Walker-A lysine and serine; Table S1) also impact upon ATP occlusion [23]. Secondly, broadly speaking, the basic communication between NBD and TMD is unaltered by the E→A mutations, with drug stimulation of ATP occlusion observed [23]. Demonstration of the occluded nucleotide in wild type P-gp would serve as additional evidence for the schematic in Fig. 4.

7. Mutations at the NBD:NBD interface (Table S2)

By definition within this section we mean the classical sandwich dimer NBD:NBD interface that has been observed structurally now in several studies of isolated NBDs and intact ABC transporters [25,29,32,34]. Essentially there are 6 points of interaction between the two NBDs (Table 2) and several of the mutations that localize to these interfaces directly impact upon ATP hydrolysis and/or binding.

Table 2
The NBD:NBD sandwich dimer interface

NBD1 residues	Location	NBD2 residues	Location	Closest approach Cα–Cα (Å)	Closest approach sidechain (Å)
404–407 [a]	Post A-loop	1161–1164	Pre-signature	5.5	2.2 (Pro 1162:Glu 406)
427–431 [b]	Walker-A	1174–1179	Signature	5.3	2.8 (Ser 429:Gly 1179)
473–477	Gln-loop	1117–1121	Gln-loop	8.8	5.3 (Glu 476:Glu 1119)
556–564	D-loop	1201–1208	D-loop	5.4	4.9 (Ser 559:Ser 1204)
586–590 [c]	His-loop	1204–1207	D-loop	4.7	3.6 (His 587:Asp 1207)
627–630	C-terminus	1273–1276	C-terminus	8.7	5.7 (Thr 630:Ala 1275)

The other interactions are essentially symmetrical i.e. between the same motif within NBD1 and NBD2. Distances are determined from the model of O'Mara et al. [55], which is based upon the Sav1866 structure in the presence of ADP [25].

[a] The equivalent NBD2:NBD1 interaction is residues 1047–1050 being close to residues 514–518.

[b] The equivalent NBD2:NBD1 interaction is residues 1070–1074 being close to residues 529–534.

[c] The equivalent NBD2:NBD1 interaction is residues 1231–1234 being close to residues 560–563.

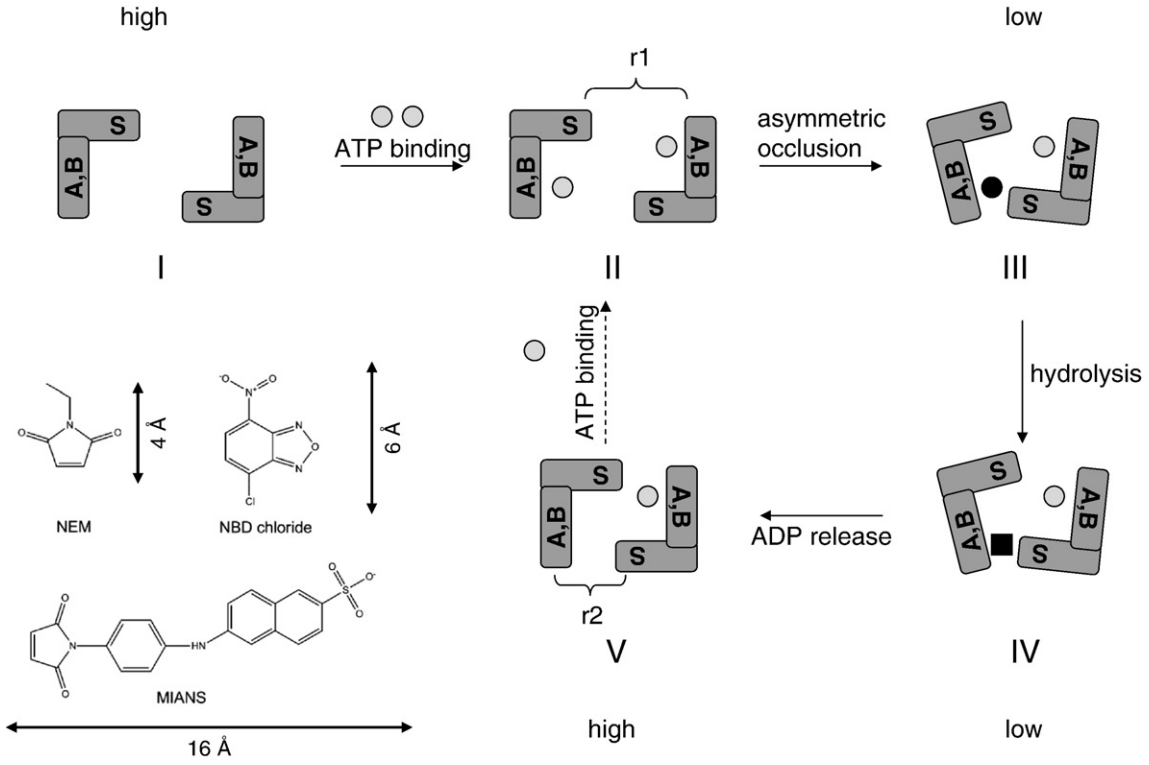

Fig. 4. Binding, and asymmetric occlusion of nucleotide in the catalytic cycle of P-gp. The two NBDs of P-gp (L-shapes) are each envisaged to bind nucleotide weakly initially and symmetrically [80], and then to progress into an asymmetric configuration in which one nucleotide is tightly bound/occluded and the other remains weakly bound. The asymmetry emerging after an initial symmetrical event is inferred [23] but supported by molecular dynamics simulations of BtuCD and MJ0796 [102,104]. The occluded nucleotide is committed to hydrolysis and the asymmetry in NBD function is likely to be imparted for subsequent cycles. The extent of NBD dimer opening remains unclear but three inhibitors of P-gp ATPase activity which interact with the Walker-A cysteine residues are depicted with their molecular sizes. Either the separation between the two NBDs prior to formation of the occluded state (r1), or subsequent to ADP release (r2), must approach the size of the largest of these, MIANS, at 16 Å [78]. The affinity for drug substrate is depicted around the perimeter with transitions occurring between configurations I and III and subsequent to configuration IV [108].

Several of the mutations at this interface (Table S2) have been made since the description of the sandwich dimer and the data already has a structural interpretation that would not differ from one presented here. For instance, several pairs of residues in the Walker-A:signature motif interface are sufficiently close that, when mutated to cysteine, they can be cross-linked at temperatures that would not necessarily support high rates of ATP hydrolysis [19]. Structurally these two regions are as close as 2.8 Å apart in a Sav1866 based model of P-gp [55] and this ability to be cross-linked is therefore to be anticipated.

However, there are some mutations that do bear further consideration. Firstly, we can revisit residues in the loop immediately C-terminal to the Walker-B motif, known as the D-loop, due to the presence of a conserved aspartic acid, 6 amino acids downstream from the "catalytic carboxylate" (Fig. 2). The neutralization of this amino acid in either half of mouse P-gp produced a protein that had a severe overall functional impediment, and 5- to 10-fold reduced ATPase activity compared to wild type [81]. However, near normal drug stimulation of ATPase activity was observed [81], suggesting that the residue is not contributing to NBD:TMD interactions. Consistent with this, it is clear from analysis of our structural templates that the D-loop makes very close contact with the His-loop and the Walker-A motif of the opposite NBD (Fig. 5a, Table 2), although comparison of NBD structures shows the D-loop to be conformationally variable [83]. We note that the two bound nucleotides are linked by as few as 7 amino acids; i.e. bound nucleotide at NBD1 is coordinated by Walker-B glutamate (residue 556), 6 amino acids C-terminal to this, the D-loop aspartate (Asp 562) is in direct contact (<4 Å) with the His-loop of NBD2 (His 1232), which itself interacts with bound nucleotide at NBD2. This apposition suggests that the reduction in overall ATPase activity is due to impaired NBD:NBD communication, potentially involving a direct charge:charge interaction with the "switch histidine" residue [32,39].

We also consider an alternative interpretation for mutations made to the second glycine of the signature motif (residues 534 and 1179; Fig. 5b). The consecutive glycine residues in the signature are generally considered to be conserved on structural grounds, the flexibility of the glycine backbone being a requirement to maintain the initial α-helical turn of the long post-signature motif α-helix (α5). That is, the glycine residues are conserved because they are intrinsic to the structure of the NBD. Indeed, mutation of this residue to either aspartic acid, serine or valine abrogates ATPase activity (and in some instances expression level) [84,85]. Examination of a P-gp model based upon the Sav1866 structure indicates that three alternative explanations can be considered for these effects. Firstly, as theorized in

the literature, the glycine may be required for intrinsic structural integrity of the NBD. The occurrence of two glycine residues at the 1st and 2nd positions of an α-helix is rare, and positional preference mutagenesis studies indicate that glycine at the 1st or 2nd position is unfavourable [86]. It therefore seems unlikely that a conserved di-glycine within the first turn of α-helix has a structural role. Secondly, the residue may have an indirect role to play in the hydrolysis of ATP, its nitrogen atom being positioned just 3 Å from the γ-phosphate of the bound ATP [28]. Thirdly, there may be an extrinsic (i.e. inter-domain) structural role for the glycine residues due to their proximity to the TMDs. The structural perturbation caused by mutation of the glycine

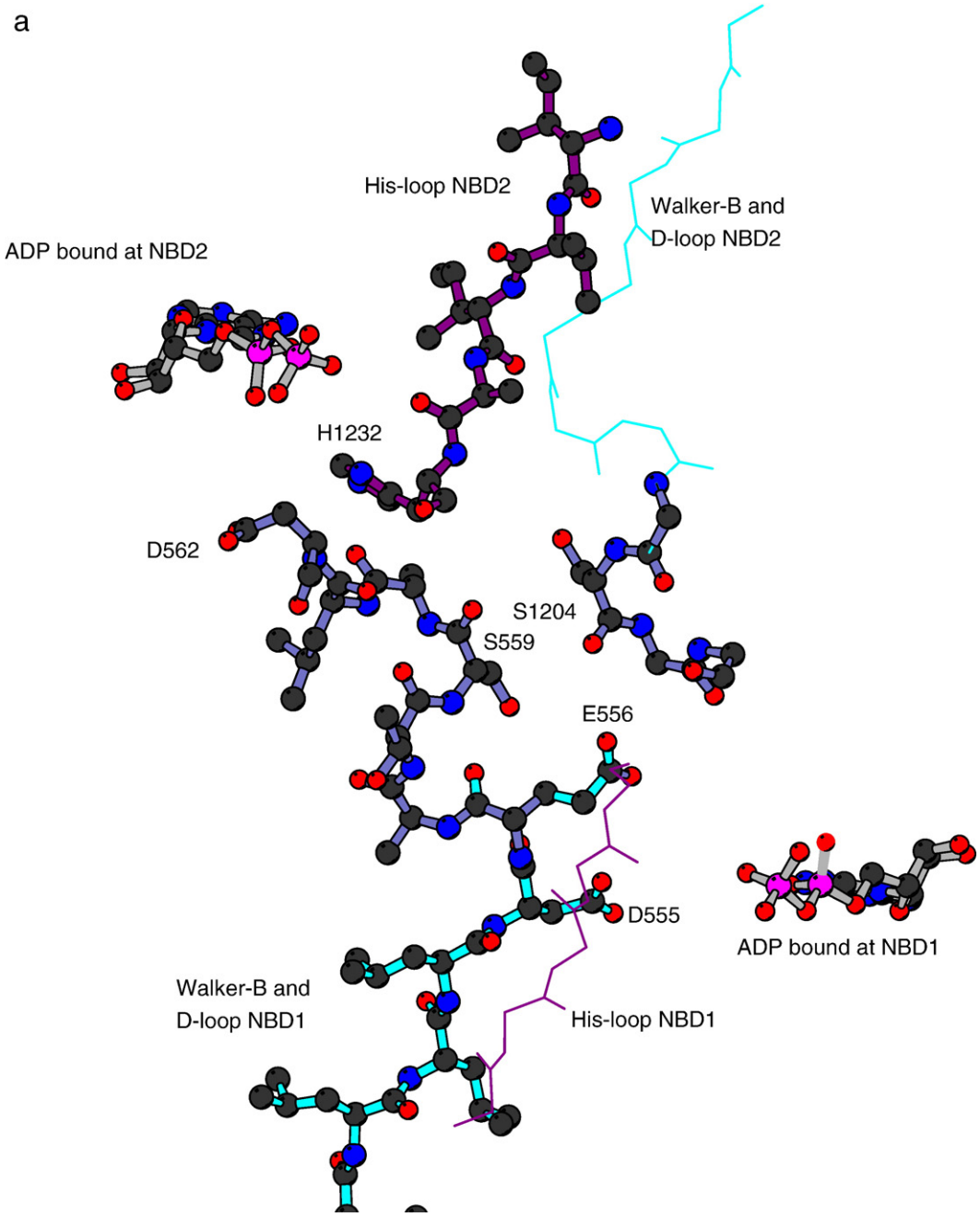

Fig. 5. Putative NBD:NBD interactions in P-glycoprotein. a. Interaction of the D-loop of one NBD with the His-loop of the opposite NBD provides an allosteric span from NBD1 to NBD2. The two ADP molecules are indicated as belonging to NBD1 or NBD2 depending on which Walker-B motif is proximal to them. The C-terminal extension to the Walker-B motif (the D-loop) of NBD1 bridges the NBD:NBD interface and contacts the His-loop of NBD2, which itself is interacting with the ADP bound at this NBD. The equivalent Walker-B and D-loop from NBD2 and His-loop from NBD1 are shown in a backbone trace for clarity, with the close approach of the D-loop serine residues (Table 2) shown in ball-and-stick format. b. The conserved glycine residues of the signature motif may have structural and functional implications. The signature motif is shown in a ribbon representation with the sandwiched ADP in ball-and-stick. The proximity of the N-atoms of the glycine residues of the signature motif suggests a role in coordination of ATP. The unusual siting of two glycines at the N-terminus of an α-helix lends credence to a role in the conformational integrity of the entire X-loop and signature motif region, with implications for the interaction with the opposite TMD, through the ICL1 (cyan). Stick and ribbon colours for the highlighted motifs mirror those in Fig. 1b.

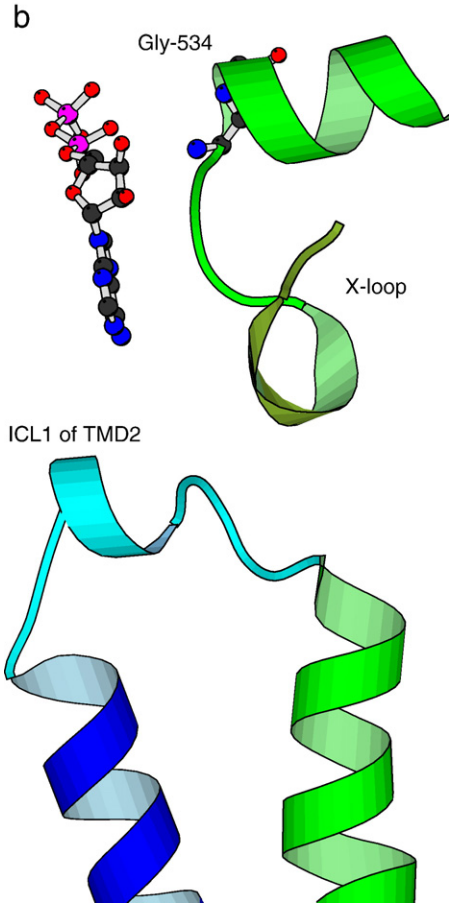

Fig. 5 (*continued*).

residue (to any residue with a sidechain) may disrupt the interaction that occurs between the α4″–α5 linker region with the intracellular loop between TM α-helices 2 and 3 (or 8 and 9) that forms part of the NBD:TMD interface [25]. This interaction is between NDB1 and TMD2 (or vice versa) and leads to reasoning that mutations in the signature motif may actually affect drug stimulated ATPase activity. Certainly, this is consistent with the experimental findings [84,85].

Finally, it is worth briefly revisiting a residue that was employed in a cysteine-scanning mutagenesis screen to document the accessibility residues within P-gp NBD1 [87]. Lysine 515, which when mutated to cysteine is accessible to biotin maleimide in permeabilized cells, is located N-terminal to the signature motif and is in close apposition to two loops in the opposite NBD, just C-terminal to the A-loop, and beyond the final conserved motif (the His-loop) in the NBD. The function of this single cysteine isoform in a whole cell transport assay was unaffected [87] but our structural analysis places this residue at the NBD:NBD interface and indicates that further mutagenesis at this part of the interface may identify the existence of key functional interactions contributing to NBD:NBD co-operativity.

One issue that continues to emerge from such studies is the degree to which the NBDs of P-gp are functionally symmetric [88,89]. Clearly this issue may not even be resolved if we have a structure of P-gp as structural asymmetry within its NBDs is to be expected as they are only 60% identical at the amino acid level. A rational explanation is that, if there is a functional asymmetry within the NBDs of P-gp, it may be rather more subtle than for instance the asymmetry displayed by the two halves of the TAP transporter, the two homologous halves of MRP1 and the two homologous halves of the cystic fibrosis conductance transmembrane regulator (CFTR) [90–92]. In these latter instances functional asymmetry might be anticipated by analysis of the key sequence motifs and the NBD sandwich dimer. As Gaudet have pointed out for TAP1/2 for example, only one of the two ATP binding sites formed by dimerization of TAP1 and TAP2 presents what might be called a consensus ABC transporter ATPase site. The other contains substitutions as both the Walker-B and the His-loop which could render this site less/non-functional [28]. In contrast, P-gp contains all the key sequence elements necessary to have two consensus ABC ATPase sites (Fig. 2).

8. Mutations at the NBD:TMD interface (Table S3)

Residues that have been mutated in P-gp studies can now be more reliably interpreted if they have an effect on NBD:TMD coupling, due to the advances made in understanding intact ABC exporters [25]. This interface could not be reliably determined from previous structures of NBD dimers, or from attempts to use the ABC importer structures as templates for understanding domain:domain interaction in P-gp, such is the low structural conservation between ABC exporters and importers in this region [25,27,36]. Additionally, mapping onto the initial structures of MsbA is also no longer justified, but the revised structures will enable a comparison to be made with the interpretations that result from consideration of a Sav1866 based model of P-gp [55]. Among the many novel observations in the Sav1866 structure was the close contact between residues

Table 3
The TMD:NBD interface in P-gp

NBD residues	Region of the NBD	TMD residues	Region of the TMD	Closest approach Cα–Cα (Å)	Closest approach sidechain (Å)
442–445 (1085–1088)	Post Walker-A	157–161 (798–802)	ICL1	6.4	3.1 (Tyr 444:Gly 161)
442–445 (1085–1088)	Post Walker-A	907–913 (262–268)	ICL2	8.3	4.0 (Leu 443:Val 908)
478–483 (1121–1126)	Post Gln-loop	899–903 (256–260)	ICL2	6.2	2.1 (Phe 480:Glu 899)
490–493 (1133–1136)	Helical domain	905–911 (262–268)	ICL2	7.7	1.5 (Tyr 490: Leu 910)
524–528 (1167–1171)	X-loop	805–809 (164–168)	ICL1	5.0	3.2 (Gly 525:Asp 805)

For each TMD:NBD contact region the residues contributing to the interaction are given for one NBD:TMD interface, with the opposite, equivalent interface given in parenthesis in columns 1 and 3. Distances are determined from the model of O'Mara et al. [55], which is based upon the Sav1866 structure in the presence of ADP [25].

in TMD1 and NBD2 and vice versa (Table 3). The most striking mapping of residues at the NBD:TMD interface comes from a recent cross-linking study of P-gp, designed to test whether the domain interaction observed in Sav1866 [25] is also apparent in P-gp [93]. Residues 443 (just a few amino acids after the end of the Walker-A motif) and 909 (in the intracellular loop between TM helix 8 and 9) in a cysteine-less version of P-gp were mutated to cysteine and were shown to form a cross-link in the presence of various bifunctional maleimides [93]. Similarly, residue 474, immediately N-terminal to the Gln-loop could be

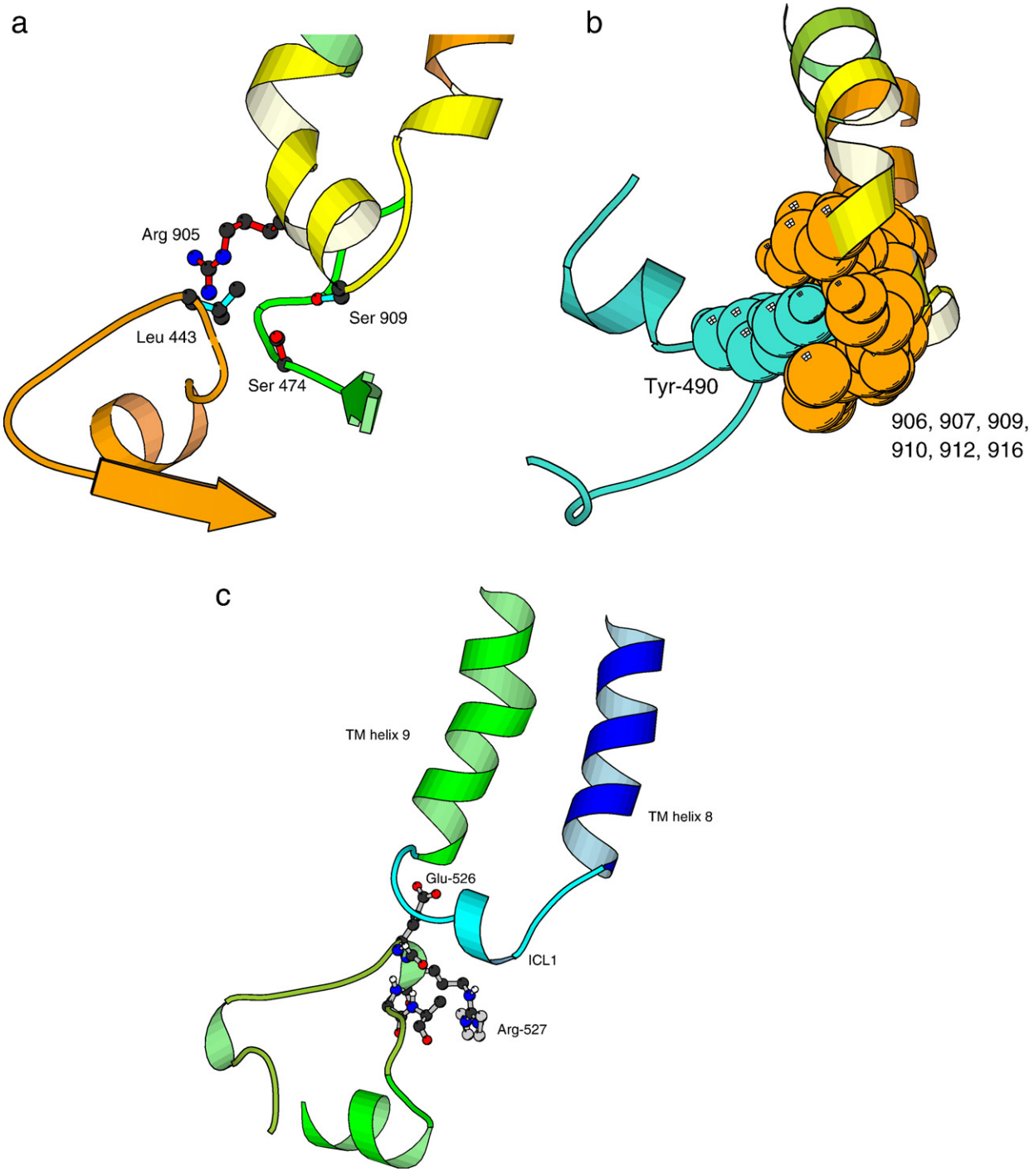

Fig. 6. Proposed interactions between the NBD and TMD of P-glycoprotein. a. Cross-linking provides evidence that the domain swapping observed in Sav1866 is a feature of P-gp. Residue 443 in NBD1 can be cross-linked to residue 909 following mutation to cysteine (cyan pair) as can residues 474 and 905 (red pair). b. Spacefill representation of the packing of Tyr 490 against the sidechains of residues in ICL2. Tyrosine 490, the equivalent residue to Phe 508 in CFTR is in close proximity to the opposite intracellular helix. The loop bearing Tyr 490 is shown in cyan in cartoon representation, with Tyr 490 itself rendered as spacefill. The TM10, ICL2, and TM11 elements are coloured green, yellow and orange respectively. c. The ERGA sequence of one NBD, which is part of the X-loop interacts with the opposite TMD, through the 1st intracellular coupling loop. The intracellular extensions to TM helices 8 and 9 and their connecting region (ICL1) are shown in cartoon fashion, as is the X-loop of the opposite NBD. The ERGA sequence is shown in ball-and-stick representation.

cross-linked to residue 905 when both were mutated to cysteine [93] (Fig. 6a). The fact that cross-linking was observed with short spacer-length reagents indicates that the residues are proximal in the structure — providing credence that P-gp has the same domain architecture as that observed in Sav1866 [93].

Tyrosine 490 has been discussed in relation to P-gp due to its analogy with the common cystic fibrosis mutation, deletion of Phe 508. This mutation in CFTR causes defective synthesis and trafficking of the protein [94]. Introduction of a single amino acid deletion at Tyr 490 also showed an important role in the normal trafficking and function of P-gp [95,96]. Structural analysis of tyrosine 490, which resides in NBD1, shows a striking proximity to the 2nd intracellular coupling helix of the opposite NBD, i.e. the region connecting TM helices 10 and 11 in a P-gp model [25,55], and is therefore a point of interaction between NBD and TMD (Fig. 6b). On closer inspection, tyrosine 490 is seen to contact two residues in the coupling helix, Thr 906 and Leu 910, as well as being within 4 Å of other residues in the 906–916 region. These residues evidently form a "pocket" into which Tyr 490 appears to fit tightly (Fig. 6b). The disruption of this tight NBD:TMD interaction may well correlate with the experimental data showing reduced expression of the protein [95,96], presumably due to significant effects on the folding pathway of P-gp. Notably, this tyrosine residue is conserved in both NBDs of human and mouse P-gp as well as in TAP1 (Fig. 2).

The domain swapping evident in Sav1866 also occurs between the intracellular linker between TM α-helices 4 and 5 (ICL2) and stretches of residues in the opposite NBD within what is known as the X-loop. Remarkably, evidence of the importance of this stretch of residues has been in the literature for over a decade. Beaudet et al., comparing the sequence of the N- and C-terminal NBDs of mouse MDR3 demonstrated that substitution of residues 522–525 (ERGA; equivalent residue numbers are 526–529 in human P-gp) from NBD1 with residues 1167–1170 from NBD2 (DKGT, 1171–1174 in human P-gp) led to drastic alterations in the ability to confer resistance to several anti-cancer drugs, and the ability to complement the *Ste6* transporter in *S. cerevisiae* [97]. The structure of Sav1866 [25] now shows that these residues are in contact with residues in ICL1 reinforcing the observation that specific residues within the X-loop of NBD 1 are required for productive NBD:TMD interactions [97,98] (Fig. 6c). In contrast, a further residue that could not be substituted between NBD1 and NBD2 in mouse MDR3 (Thr 578, Thr 582 in P-gp) is not at a NBD:TMD boundary, and no explanation for this effect is apparent from examination of the structural data.

Several further residues mutated to cysteine by Blott et al. [87] reinforce just how functionally malleable the TMD:NBD interface must be. Ser 474 and Ala 529 can both be mutated to cysteine without loss of overall transport function, and both have interdomain interactions of less than 5.0 Å with residues in the TMD.

The final NBD loop we discuss is the Gln-loop, the eponymous glutamine of which is almost strictly conserved in ABC transporters [10]. Urbatsch et al. described the results of the

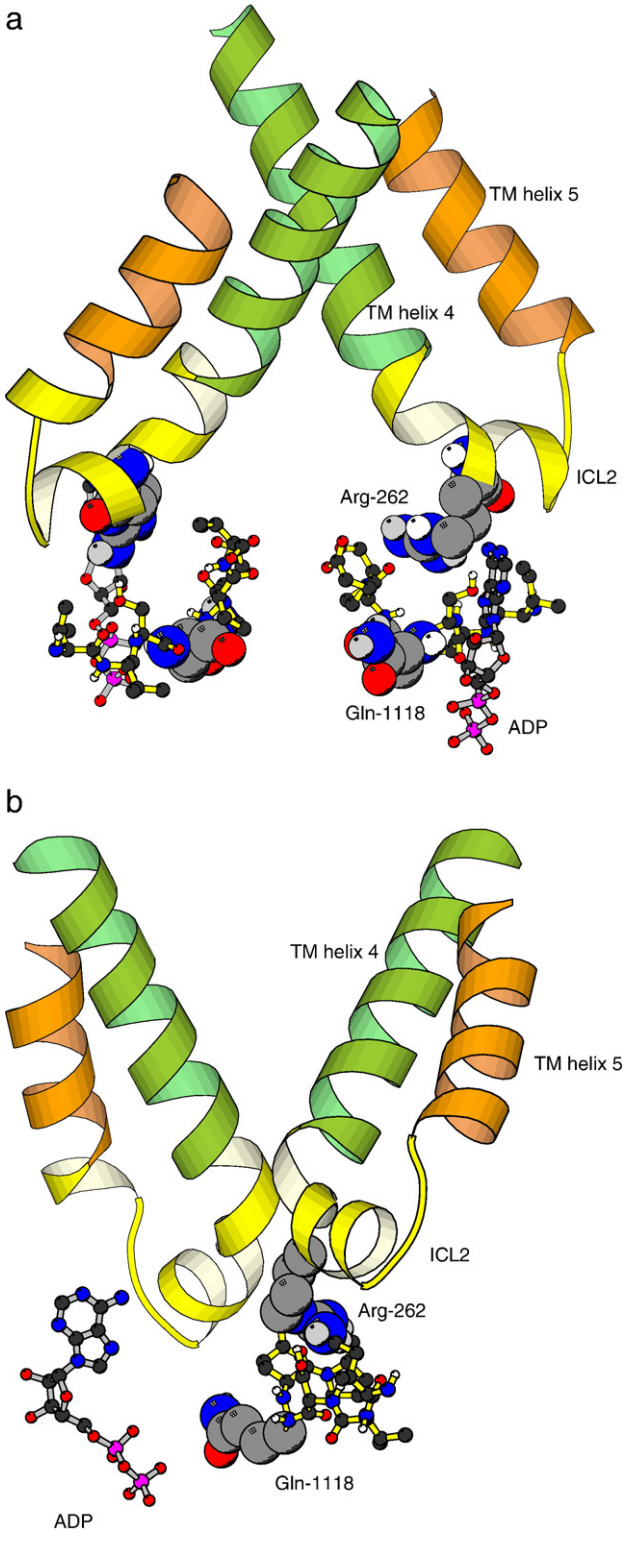

Fig. 7. The Gln-loop may be involved in multiple allosteric processes within P-gp. a. The Gln-loop of one NBD is capable of interactions with bound nucleotide, the opposite Gln-loop and with the 2nd ICL of the opposite TMD. The conserved glutamines (475 and 1118) are shown in spacefill representation, together with the closest amino acid in the opposite ICL (arginines 262 and 905). The TM helices preceding (TM4/10, green) and following (TM5/11, orange) the intracellular loop (yellow) are shown in ribbon representation. The rest of the Gln-loop and the bound nucleotide are in ball-and-stick configuration. One of the ADP molecules lies in front of the plane in this view, and the other lies behind the plane. b. Rotation through 90° allows clearer inspection of the possible interactions. For clarity, only Q1118, R262 and one nucleotide are shown.

single mutants of residues Q475 and Q1118 to either a glutamate or an alanine residue [89]. The mutations were seen to affect the fold-simulation of ATPase activity by certain drugs, with Q→A mutations more severely disrupted compared to Q→E mutants, implying a role for this residue in TMD:NBD communication [89], consistent with predictions from molecular dynamics simulations of HisP [99,100]. In agreement with this, the location of residues Q475 and Q1118 in our Sav1866 based homology model is significantly close (less than 6 Å) to the sidechains of arginines 905 and 262 respectively, in the ICL2 (Fig. 7). However, two alternative interpretations also present themselves. The first is correlated with the observations that the experimental mutations caused a 10-fold reduction in the basal ATPase activity (i.e. the activity in the absence of drug substrate). This activity is believed to be due to NBD:NBD communication only [101], and intriguingly the two Gln residues lie close to the NBD:NBD boundary as well. Indeed, the adjacent glutamate residues are as close at 6 Å indicating that residues in the Gln-loop could contribute to NBD:NBD interactions as well. A further role for the Gln-loop is in the direct interaction with nucleotide as the residue is also ca. 6 Å from bound triphosphate nucleotide [26]. Again a disruption of the basal ATPase activity following mutation of the conserved Gln would be consistent with this hypothesis. It seems that the Gln-loop (which has so far escaped much experimental mutagenesis) may be able to play functional roles in *intra*-domain communication (i.e. the interaction with ATP bound to the same NBD), *inter*-domain NBD:NBD communication and *inter*-domain NBD:TMD communication.

9. Perspectives

There is no doubt that mutagenesis still has a significant role to play in determining the mechanism of P-glycoprotein, and by inference, other ABC transporters. There is sometimes a tendency to dismiss the results of site-directed mutagenesis as being due to a gross disruption to the protein structure, rather than a specific effect on protein function. However, we have not felt that this is necessary in the current work — most of the mutations we have analysed have effects that can be rationalised in terms of protein structural models. Moreover, the accumulating structural data on related proteins now enables both a more targeted mutagenesis approach and a more rational interpretation of existing and new mutagenesis data. In particular, the structural data on Sav1866 [25] and the revised MsbA structures [115] will enable much mutagenesis at the NBD:TMD interface. In addition, the emergence of structural data for ABC transporters at different stages of the catalytic cycle may enable further investigations of residues at the NBD:NBD interface. We have highlighted here at least two regions (the Gln-loop and the His-loop), mutations within which and proximal to, may continue to yield mechanistic insights in P-glycoprotein function. Unequivocal interpretations may still be a rarity, particularly when a mutation affects the communication between domains within the transporter. In order to redress this we need to provide a fully structural and mechanistic understanding of P-gp by using a full arsenal of biochemical and biophysical techniques. Electron microscopy has pushed our understanding of P-gp structure to almost secondary structural resolution and has enabled the protein to be imaged in the presence and absence of nucleotide [47]. Combining this data with emerging crystallographic data on related ABC transporters [25] may soon provide homology models for P-gp that are a good first approximation to structures at different stages of the catalytic cycle. Similarly, the deployment of molecular dynamics simulations may be able to bridge the gulf between static structural data/models and the changes in protein conformation and inter-domain interfaces that are proposed to be relevant to P-gp function [102–104]. Such dynamic changes may be further explored by using electron paramagnetic resonance (EPR) and solid state NMR spectroscopy. One EPR study for P-gp has been reported [105] and several exist for MsbA [106,107]. The interpretation of EPR data, in terms of its ability to report on local protein dynamics will undoubtedly complement and make a considerable addition to our understanding of this protein. For example, although mutations in the Walker-A motif may seem to be fully explored, EPR spectroscopy could reveal the changes occurring at the NBD:NBD interface in terms of residue exposure, local polarity and local conformational flexibility.

So the current body of data we have analysed here cannot in isolation explain the catalytic mechanism of P-gp and the accompanying changes in inter-domain interaction. However, we hope that with this analysis we have provided a useful synopsis of the field and that the growing database of P-gp NBD mutations may continue to spark further investigations.

10. Note added in proof

The structures of MsbA have been republished [115]. A full comparison of these structures will appear at the NBD mutation database website. The existence of an occluded nucleotide state in wild type P-gp has been recently documented [116] adding further credence to the model in Fig. 4.

Acknowledgements

IDK thanks the P-gp community for its interest in the NBD mutation database over the past several years, and for continuing discussions pertaining to some of the mutations described.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2007.10.021.

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