The Packing of the Transmembrane Segments of Human Multidrug Resistance P-glycoprotein Is Revealed by Disulfide Cross-linking Analysis*

(Received for publication, December 9, 1999, and in revised form, January 4, 2000)

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Residues from several transmembrane (TM) segments of P-glycoprotein (P-gp) likely form the drug-binding site(s). To determine the organization of the TM segments, pairs of cysteine residues were introduced into the predicted TM segments of a Cys-less P-gp, and the mutant protein was subjected to oxidative cross-linking. In SDS gels, the cross-linked product migrated with a slower mobility than the native protein. The cross-linked products were not detected in the presence of dithiothreitol. Cross-linking was observed in 12 of 125 mutants. The pattern of cross-linking suggested that TM6 is close to TMs 10, 11, and 12, while TM12 is close to TMs 4, 5, and 6. In some mutants the presence of drug substrate colchicine, verapamil, cyclosporin A, or vinblastine either enhanced or inhibited cross-linked. Cross-linking was inhibited in the presence of ATP plus vanadate. These results suggest that the TM segments critical for drug binding must be close to each other and exhibit different conformational changes in response to binding of drug substrate or vanadate trapping of nucleotide. Based on these results, we propose a model for the arrangement of the TM segments.

The multidrug resistance P-glycoprotein (P-gp); product of the human MDR1 gene) uses energy from ATP hydrolysis to pump a broad range of cytotoxic compounds out of the cell. It is found in the plasma membrane of cells lining the gastrointestinal tract, the brush border of renal proximal tubules, on the biliary face of hepatocytes (1), or on the luminal surface of capillary endothelial cells of the brain and testes (1, 2). The location of P-gp in tissues, together with studies on P-gp

“knock-out” mice suggest that P-gp likely protects the organism from toxic xenobiotics (3). P-gp is clinically important because it contributes to the phenomenon of multidrug resistance during AIDS (4) and cancer chemotherapies (reviewed in Ref. 5). The protein is a member of the ATP-binding cassette family of transporters (6). The 1280 amino acids of P-gp are organized in two tandem repeats of 610 amino acids that are joined by a linker region of 60 amino acids (7, 9).

The mechanism that allows P-gp to recognize such a broad range of compounds is unknown. Both halves of P-gp are required for substrate-stimulated ATPase activity (10) and for drug binding (11). It was recently shown that the TM domains alone could mediate drug binding (12). A deletion mutant lacking both nucleotide-binding domains could still interact with drug substrates. The drug-binding site(s) in P-gp likely consists of residues from multiple TM segments (13). Understanding the mechanism of drug recognition by P-gp will require knowledge about the packing of the TM segments and their response to the presence of different substrates.

In this study, the packing and flexibility of the TMs of P-gp were examined by disulfide cross-linking analysis. Pairs of cysteine residues were introduced into the predicted TM segments of a mutant Cys-less P-gp. The mutant proteins were assayed for disulfide cross-linking.

EXPERIMENTAL PROCEDURES

Construction of Mutants—Cysteine residues were introduced into the cDNAs of either Cys-less NH2-half or COOH-half molecules of Cys-less P-gp containing the epitope for monoclonal antibody A52 (14) or into full-length Cys-less P-gp with a (His)10 tag at the COOH terminus (15). The presence of a histidine tag facilitated purification of the mutant P-gp by nickel-chelate chromatography (16). Sulfhydryl Cross-linking, Purification of P-gp Mutants, and Measurement of Drug-stimulated ATPase Activity—Membranes were prepared from HEK 293 cells expressing mutant P-gp that was grown in the presence of 10 μM cyclosporin A (16, 17) and subjected to oxidative cross-linking as described previously (15). Purification of histidine-tagged P-gp by nickel-chelate chromatography and measurement of drug-stimulated ATPase activity was described previously (16). The effect of cross-linking on ATPase activity was done as described previously (15). Immunoblot analysis was with monoclonal antibody A52 or with rabbit polyclonal antibody (18).

RESULTS

Cross-linking of P-gp Mutants—We previously showed that predicted TM6 and TM12 of P-gp are close to each other because mutant P350C(TM6/S993C(TM12) was cross-linked after exposure to oxidant (15). This mutant was quite interesting because of concomitant loss of drug-stimulated ATPase activity upon cross-linking. This effect could be reversed by reducing agents such as dithiothreitol. If the TM segments of P-gp possess enough flexibility to accommodate substrates of varying sizes, then it was reasonable to assume that residues in other TMs could be cross-linked to P350C or S993C. Therefore, two points were considered when selecting the combination of mutants for cross-linking analysis. The first point was that the assay for the presence of cross-linked product relied on the cross-link causing changes in the mobility of the protein on SDS-PAGE. The formation of disulfide bonds between widely separated residues is more likely to alter the electrophoretic mobility of proteins than between two closely spaced residues. This characteristic has also been observed during cross-linking studies on the SERCA1 Ca2+-ATPase (19). A second consider-
cross-linking analysis of P-gp

| Cross-linking of S993C (TM12) with residues in the following TM: |
|---------------------------------------------------------------|
| TM1          | TM2          | TM3          | TM4          | TM5          |
| M51C         | Y130C        | G185C        | G226C        | I936C        |
| V72C         | I131C        | I186C        | L227C        | T294C        |
| V53C         | Q132C        | G187C        | S228C        | A295C        |
| G54C         | S134C        | K189C        | A230C        | 1297C        |
| T55C         | F135C        | I190C        | A239C        | 1298C        |
| L56C         | W232C        | W233C        | S298C        | +            |
| A57C         | C137C        | M192C        | A233C        | G300C        |
| A58C         | L138C        | F193C        | K234C        | A301C        |
| I59C         | A139C        | F194C        | I235C        | A302C        |
| I60C         | A140C        | Q195C        | L236C        | F303C        |
| H61C         | G141C        | S196C        | S257C        | I304C        |

| Cross-linking of P350C (TM6) with residues in the following TM: |
|---------------------------------------------------------------|
| TM7          | TM8          | TM9          | TM10         | TM11         |
| F711C        | F770C        | A828C        | I867C        | A935C        |
| V712C        | F771C        | I829C        | I868C        | H936C        |
| V713C        | F772C        | G830C        | A669C        | I937C        |
| G714C        | Q773C        | S831C        | I870C        | F938C        |
| V715C        | G774C        | R832C        | A871C        | G939C        |
| F716C        | F775C        | L833C        | G872C        | I940C        |
| C717C        | T776C        | A834C        | V873C        | T941C        |
| A718C        | F777C        | V835C        | V874C        | F942C        |
| F719C        | G778C        | E875C        | E876C        | S943C        |
| I720C        | K779C        | T837C        | M876C        | F944C        |
| N721C        | A780C        | Q838C        | K877C        | T945C        |
| G722C        | G781C        | N839C        | M878C        | Q946C        |
| G723C        | E782C        | I840C        | L797C        | A947C        |

* a — no cross-linked product detected in SDS-PAGE.
* b -- cross-linked product detected in SDS-PAGE.

ation was that P350C (TM6) and S993C (TM12) are predicted to be close to the cytoplasmic side of the membrane. Therefore, the cysteine residues introduced into the other TMs should also be close to the cytoplasmic side of the membrane. Accordingly, mutants were constructed that had either P350C (TM6) and cysteines introduced into predicted TMs 7 to 11, or between S993C (TM12) and a cysteine introduced into predicted TMs 1 to 5. A total of 125 different mutants were constructed for activity and cross-linking analysis (Table I). All mutants yielded the mature (170 kDa) P-gp as the major product when expressed in HEK 293 cells. It was important that only one cysteine residue was introduced into their respective half-molecules. A representative result is shown in Fig. 1B. Cross-linking was not detected when only one cysteine was present. By contrast, when the appropriate cysteine residues were present in the half-molecules, a cross-linked product with slower mobility was detected on SDS gels.

**Effect of Temperature on Cross-linking**—P-gp mutants that could be cross-linked at 37 °C were then tested to see if cross-linked products could be detected at lower temperatures (21 °C and 4 °C). Table II shows the minimum temperature required for cross-linking was 4 °C for mutants A233C(TM4)/S993C(TM12), 1235C(TM4)/S993C(TM12), L236C(TM4)/S993C(TM12), and I299C(TM5)/S993C(TM12); 21 °C for mutants V231C(TM4)/S993C(TM12), W232C(TM4)/S993C(TM12), A295C(TM5)/S993C(TM12), V874C(TM10)/P350C(TM6), M876C(TM10)/P350C(TM6), and G993C(TM11/P350C(TM6); and 37 °C for mutants L227C(TM4)/S993C(TM12) and E875C(TM10/P350C(TM6)).

**Effect of Drug Substrates on Cross-linking**—Drug substrates can interact with the TM segments and alter their structure (12, 13, 20). The 12 P-gps that were cross-linked were treated with oxidant in the presence drug substrate colchicine, verapamil, cyclosporin A, or vinblastine. In these cross-linking experiments, the amount of oxidant was lowered by 10-fold (0.2 mM), and the minimum temperature required to induce cross-
linking was used so that subtle effects were not obscured. A representative result (mutant G939C/P350C) is shown in Fig. 1C. In this mutant, cross-linking was decreased in the presence of verapamil or vinblastine, but not in the presence of colchicine or cyclosporin A. The results for the other mutants are summarized in Table III. Cyclosporin A inhibited the cross-linking of mutants L227C(TM4)/S993C(TM12), W232C(TM4)/S993C(TM12), I299C(TM5)/S993C(TM12), and E875C(TM10)/P350C(TM6), but inhibited that of mutant G939C(TM11)/P350C(TM6). Vinblastine enhanced the cross-linking of mutant L227C(TM4)/S993C(TM12) and inhibited that of mutants I299C(TM5)/S993C(TM12), E875C(TM10)/P350C(TM6), and G939C(TM11)/P350C(TM6).

**Effect of ATP on Cross-linking**—Hydrolysis of ATP can also alter the structure of the TM domains of P-gp (15). The effects of ATP and vanadate on the cross-linking pattern of the 12 positive mutants were analyzed. Vanadate was included because it “locks” P-gp in an inactive conformation when it occupies one of the nucleotide-binding sites with ADP (21). The results of a representative mutant G939C(TM11)/P350C(TM6) are shown in Fig. 1B. Cross-linking (oxidant (0.2 mM) at 37 °C) of this mutant was inhibited when ATP plus vanadate were present. Similar results were obtained with mutants L227C(TM4)/S993C(TM12), W232C(TM4)/S993C(TM12), W232C(TM4)/S993C(TM12), I299C(TM5)/S993C(TM12), E875C(TM10)/P350C(TM6), and G939C(TM11)/P350C(TM6) (Table III). Therefore, inhibition by ATP plus vanadate decreases the conformational flexibility of the TM segments.

**Effect of Cross-linking on Drug-stimulated ATPase Activity**—It was of interest to determine whether cross-linking between the various TM segments inhibited or stimulated drug-stimulated ATPase activity. It is possible that the ATPase activity of P-gp may be uncoupled from drug-binding upon cross-linking. Accordingly, membranes were prepared from HEK 293 cells expressing the mutant P-gps and treated with (2 mM) or without (None) ATP and vanadate for 10 min at 37 °C. The reaction was stopped by addition of EDTA and the oxidant removed by dilution with an excess of Triis-buffered saline buffer, followed by centrifugation of the treated membranes. Purified histidine-tagged P-gp was assayed for verapamil-stimulated ATPase activity after addition of lipid. Oxidative cross-linking inhibited the verapamil-stimulated ATPase activity of all mutants except Cys-less P-gp. Mutants L227C/S993C, V231C/S993C, W232C/S993C, A233C/S993C, I235C/S993C, L236C/S993C, A295C/S993C, I299C/S993C, V874C/P350C, E875C/P350C, M876C/P350C, and G939C/P350C were inhibited by 81, 88, 90, 93, 81, 78, 87, 77, 70, and 78%, respectively. When the cross-link-induced P-gps were treated with 5 mM dithiothreitol, greater than 70% of their original activities were restored (when compared with the activity of P-gp isolated from membranes not treated with oxidant). These results suggest that the active forms of P-gp can be cross-linked.

**DISCUSSION**

The results from this and previous studies (14, 15) suggest that TMs 4, 5, and 6 in the NH2-terminal half are close to TMs 10, 11, and 12 in the COOH-terminal half of P-gp. Disulfide cross-linking can occur between residues in TM6 and TMs 10, 11, and 12 and between residues in TM12 and TMs 4, 5, and 6.

Our results suggest that binding of substrate induces conformational changes in the TM segments. Colchicine, verapamil, cyclosporin A, and vinblastine had different effects on the cross-linking pattern. A transporter with restricted substrate specificity would be expected to show limited conformational changes upon substrate binding. The capacity of P-gp to accommodate such structurally different substrates could partly be due to relative flexibility and fluidity of TM domains. This is consistent with the finding that there is considerable variability in the ability of colchicine, verapamil, cyclosporin A, or vinblastine to stimulate the ATPase activity of P-gp (13) or to induce conformational changes in the nucleotide-binding sites (22). The fluid nature of the TM segments is suggested by

**TABLE II**

| Residues   | TM segments | 4°C | 21°C | 37°C |
|------------|-------------|-----|------|------|
| L227C/S993C | 4/12        | —   | +    | +    |
| V231C/S993C | 4/12        | —   | +    | —    |
| W232C/S993C | 4/12        | +   | +    | +    |
| A233C/S993C | 4/12        | —   | +    | +    |
| I235C/S993C | 4/12        | +   | +    | +    |
| L236C/S993C | 4/12        | +   | +    | +    |
| A295C/S993C | 5/12        | —   | —    | +    |
| I299C/S993C | 5/12        | —   | —    | +    |
| V874C/P350C | 10/6        | +   | +    | +    |
| E875C/P350C | 10/6        | +   | +    | +    |
| M876C/P350C | 10/6        | —   | —    | —    |
| G939C/P350C | 11/6        | —   | +    | +    |

| a | no cross-linked product detected in SDS-PAGE. |
| b | +, cross-linked product detected in SDS-PAGE. |
the finding that only 4 of the 12 mutants could be cross-linked at 4 °C (Table II), while all were cross-linked at 37 °C.

Vanadate plus ATP inhibited cross-linking of the mutants by locking-in the nucleotide-binding domain(s) of P-gp in a fixed conformation (Table III). The rigid nature of the locked-in conformation is then transmitted to the TM domains. This supports the notion that there is "cross-talk" between the nucleotide-binding domains and the TM domains and that ATPase activity is directly linked to drug-P-gp interaction.

We have proposed a working model of the arrangement of the TM segments of P-gp (Fig. 2). An interesting feature about the model is that the TMs involved in cross-linking have been reported to be important for P-gp-drug interactions. Cysteine-scanning mutagenesis of the TM segments and labeling with a thiol-reactive substrate, dibromobimane, provided direct evidence for interaction of substrate with residues in TMs 6, 11, and 12 (13, 20). Dibromobimane reacted with specific cysteine residues in TMs 6, 11, and 12 and inhibited the activity of P-gp. The presence of substrates such as colchicine, verapamil, or vinblastine inhibited labeling by dibromobimane. Many mutations in TMs 4, 5, and 6, and in TMs 10, 11, and 12, have been reported to alter the substrate specificity or activity of the transporter (23–31). Labeling studies with photoactive analogs of P-gp substrates also suggest that TMs 4, 5, and 6 and TMs 10, 11, and 12 may participate in drug-protein interactions (32–36). The model in Fig. 2 also takes into consideration that no cross-linked products were detected between TMs 1 to 3 and TMs 7 to 9 when cysteine residues were introduced into the full-length Cys-less P-gp or into the Cys-less half-molecules (data not shown).

In summary, the TM segments postulated to be involved in drug binding are quite close to each other on the cytoplasmic side of the membrane. The TM segments and nucleotide-binding domains appear to be conformationally flexible to be able to accommodate structurally different substrates.

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