Photoaffinity labelling of P-glycoprotein catalytic sites

Banumathi Sankaran*, Sumedha Bhagat, Alan E. Senior

Department of Biochemistry and Biophysics Box 712, University of Rochester Medical Center, Rochester, NY 14642, USA

Received 10 September 1997

Abstract Photoaffinity labelling of hamster P-glycoprotein was carried out after trapping of radioactive Mg-8-azido-ADP in the catalytic sites by vanadate or beryllium fluoride. With either trapping agent the same labelled peptide was obtained in homogeneous form, with the sequence -FNEVVFNxPTRPDI-, corresponding to residues 1034–1037 in the C-terminal nucleotide binding site. The missing residue 'x' corresponds to Tyr-1041, which is therefore a primary reaction target of 8-azido-ADP. This tyrosine is conserved in all hamster, mouse and human P-glycoproteins. A second major labelled peptide fraction was also identified. The major sequence in this fraction was -NIHFSxPSR-, corresponding to residues 393–401 of hamster P-glycoprotein, where 'x' corresponds to Tyr-398 in the N-terminal nucleotide binding site. Therefore Tyr-398, which is also conserved in other P-glycoproteins, is also a reaction target for 8-azido-ADP. In sequence alignment of the two nucleotide binding sites, Tyr-398 exactly corresponds to Tyr-1041. The data indicate that these two tyrosines lie close to the adenine ring of bound substrate MgATP in the respective catalytic sites of P-glycoprotein.

Key words: Multidrug-resistance; P-glycoprotein; Catalytic site; Photoaffinity labelling

1. Introduction

P-glycoprotein (Pgp), also called multidrug-resistance protein, is a plasma membrane located glycoprotein. In vitro it has been shown to confer multidrug-resistance phenotype on cells. The mechanism is not yet understood in detail, but it is clear that Pgp has the capability of excluding drugs from cells in an ATP-dependent manner. The most commonly considered current hypothesis is that Pgp acts as an ATP-driven drug-export pump. Multidrug-resistance is an important obstacle in treatment of human cancer, and there is considerable interest in the potential role of Pgp in rendering tumor cells resistant to chemotherapeutic drugs [1–5].

Pgp molecules from human and rodents show similar amino acid sequences, and are about 1280 residues in length, with two similar but not identical nucleotide binding sites (NBS). Pgp shows substantial ATPase activity in absence of added drugs ('basal activity'), which is stimulated several-fold by drugs [6]. The catalytic sites are known to be of low affinity for MgATP ($K_M \sim 1$ mM) and of low selectivity for nucleotides [7,8]. The photoaffinity labelling analog Mg-8-azido-ATP is a good hydrolysis substrate, with K_M value of 0.50 mM [9,10].

*Corresponding author. Fax: +1 (716) 271-2683.

Abbreviations: Pgp, P-glycoprotein; NBS, nucleotide binding site; Vi, orthovanadate; BeF_x, beryllium fluoride

Photoaffinity labelling of catalytic sites in Pgp by nucleotide analogs is potentially valuable in a number of different ways. Determination of the target residue(s) reveals important information about the protein architecture. Such information also provides guidance in determining locations for specific insertion of Trp residues, which can serve as direct optical probes of catalytic site occupancy and binding properties, as recently demonstrated in F_1-ATPase [11]. Target residues for mutagenesis experiments aimed at elucidation of catalytic roles of critical residues may also be indicated.

Earlier work has indicated that radioactive 8-azido-ATP covalently labels the NBS of Pgp upon UV-activation [9,12–14]. However, because the affinity of Pgp for the analog is relatively low, the labelling stoichiometry achieved is also low unless very high concentrations of analog are used. Initial studies in our laboratory to identify target residue(s) in Pgp using radioactive analog alone were not successful. Later studies showed that substantial covalent labelling was achieved when either vanadate (Vi) or beryllium fluoride (BeF_x) was used to trap 8-azido-ADP in the Pgp catalytic sites prior to UV-activation, that concentrations of only 10–100 μM nucleotide analog were adequate, and that highly specific labelling of Pgp in plasma membranes was achieved [15–18]. In this paper we took advantage of these trapping procedures to identify residues labelled by 8-azido-ADP in Pgp catalytic sites.

2. Materials and methods

2.1. Preparation of plasma membranes

Plasma membranes were prepared from the multidrug-resistant Chinese hamster ovary cell line CR1R12 [19] as described previously [15]. The membranes contained 15–20% (w/w) of Pgp as a fraction of total membrane protein, determined as in [9].

2.2. Trapping of 8-[α-^{32}P]azido-ADP in catalytic sites of Pgp using vanadate or beryllium fluoride, followed by UV-photolysis

Plasma membranes (6.7 mg of membrane protein) were incubated in 5 ml of 40 mM Tris-Cl pH 7.4, 0.1 mM EGTA, 2.1 mM $MgSO_4$, 50 μM verapamil, 100 μM 8-azido-[α-^{32}P]ATP (53 dpm/pmol) for 20 min at 37°C in presence of either (a) 200 μM sodium orthovanadate, or (b) 1 mM NaF and 200 μM $BeSO_4$. Samples were then placed on ice in 5 ml tissue culture dishes and subjected to UV-photolysis ($\lambda = 254$ nm, 1.1 mW/cm^2, 5 min). It was confirmed by SDS-gel electrophoresis and autoradiography that under these conditions specific photolabelling of the Pgp band occurred, without discernible photocleavage of the Pgp. Calculated labelling stoichiometry in several experiments was 0.25–0.3 mol ^{32}P/mol Pgp, determined by counting of Pgp bands excised from SDS-gels.

2.3. Trypsin fragmentation, Al-chelate resin affinity column chromatography, and HPLC fractionation of peptides

Samples were first delipidated as follows. Methanol (16 ml), chloroform (8 ml) and water (10 ml) were added and samples were vigorously vortexed at 23°C, then centrifuged at 3000×*g* for 20 min at 4°C. The top layer was discarded, methanol (18 ml) was added, and the sample was vortexed and centrifuged as above. The pellet was dried at room temperature overnight, then suspended in 330 μl of

PII S0014-5793(97)01268-4

8 M urea (freshly deionised using AG501X8 resin, BioRad) plus 660 μl of 10 mM NH_4HCO_3, pH 8.0. The final pH was adjusted to 8.0 with 1 M NH_4OH. Trypsin was added at a ratio of 1/200 (trypsin/membrane protein) and digestion was allowed to proceed for 24 h at 37°C, during which time all particulate material dissolved.

Al-chelate resin affinity chromatography was carried out as described [20,21]. Labelled peptides were eluted with 50 mM ammonium acetate pH 8.0, 15 mM KH_2PO_4, counted by Cerenkov counting, pooled and concentrated at room temperature to 2 ml final volume (SpeedVac SC110, Savant).

The concentrate was applied directly to an HPLC column (Aquapore RP300 7 micron C8, 250×4.6 mm, Brownlee Labs). Elution of peptides was carried out at a flow rate of 0.5 ml/min, with 100% Buffer A (0–10 min) and then with a gradient of 0–100% Buffer B (10–70 min). (Buffer A: 0.1% trifluoroacetic acid in water; Buffer B: 0.09% trifluoroacetic acid in 70% acetonitrile/30% water.) Radioactivity in the fractions was monitored as above (see e.g. Fig. 2). Peak fractions were concentrated at room temperature (SpeedVac).

2.4. Peptide sequencing

Labelled peptides were sequenced at the University of Kentucky Peptide Sequencing Facility (Dr. Carol Beach, Director) using an Applied Biosystems Model 477A Protein Sequenator.

2.5. Materials

8-azido-[α-^{32}P]ATP was from Research Products International. Trypsin was from Promega (Sequencing grade modified trypsin, Cat. No. V5111). Tissue culture materials were from BRL Life Technology Inc.

3. Results

Previously we developed a multidrug-resistant Chinese hamster ovary cell line (CR1R12) which overexpresses Pgp. Plasma membranes isolated from these cells contain 15–20% by weight of Pgp, in the normal mammalian lipid environment [19]. We demonstrated that on incubation of Mg-8-azido-[α-^{32}P]ATP and either Vi or BeF_x with CR1R12 plasma membranes, Pgp ATPase activity is inhibited by trapping of stoichiometric amount (1 mol/mol) of Mg-8-azido-[α-^{32}P]ADP in catalytic sites [15,17]. Subsequent UV-illumination induces

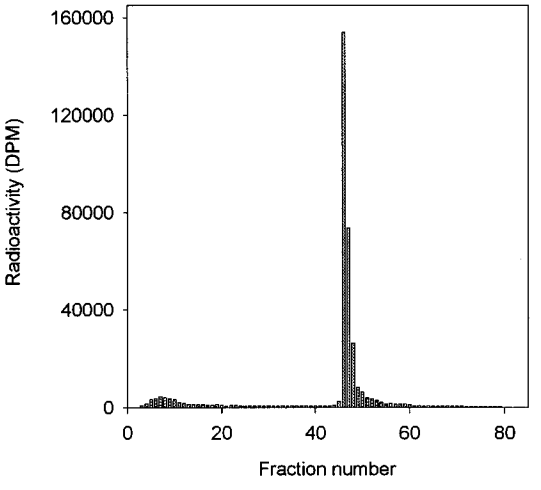

Fig. 1. Al-chelate resin affinity chromatography of labelled tryptic peptides. Pgp in plasma membranes was incubated with Mg-8-azido-[α-^{32}P]-ATP and Vi, the trapped radioactive 8-azido-ADP was photoactivated with UV-light, and the labelled membranes were digested with trypsin. The trypsin digest was applied to an Al-chelate resin affinity column. The nucleotide labelled peptides are preferentially adsorbed to this column, and elution with 15 mM KH_2PO_4-containing buffer released them (fractions 46–48). For further details see Section 2. When BeF_x was used in place of Vi, the same results were seen.

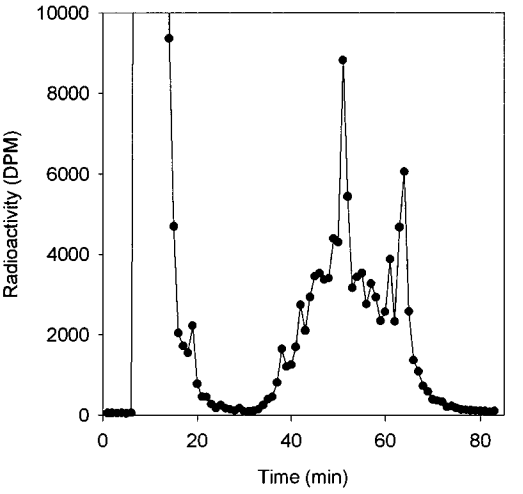

Fig. 2. HPLC analysis of the labelled peptide fraction obtained from elution of the Al-chelate resin affinity column. The radioactive peptide fraction obtained from the Al-chelate resin affinity chromatography (Fig. 1) were applied to a C8 HPLC column and eluted as described in Section 2. Radioactivity in the eluates was monitored by Cerenkov counting of the ^{32}P. Fractions at 51 and 64 min were taken for sequencing. Similar elution profiles were seen whether Vi or BeF_x was the trapping agent.

labelling of the Pgp by the trapped radioactive nucleotide. We followed these procedures here to obtained labelled Pgp.

Labelled protein was delipidated, subjected to trypsin digestion, and applied to an Al-chelate resin affinity column [20,21]. In the latter procedure the nucleotide analog labelled peptides are preferentially adsorbed to the column, and are then eluted specifically by phosphate buffer. A typical experiment is shown in Fig. 1. The radioactive fractions eluted by the phosphate buffer were pooled, concentrated and applied to a C8 HPLC column. A typical profile for elution of radioactivity from this column is shown in Fig. 2. A large, initial 'flow-through' peak was seen, followed by two distinct major radioactive fractions, eluting at 51 min and 64 min, respectively. Spectrophotometric monitoring of the HPLC column at 214 nm revealed distinct peaks at 51 and 64 min, but with a large background (data not shown). This is not unexpected since whole plasma membranes were used here as Pgp source material. For both Vi- or BeF_x-trapped samples, the same elution profiles were seen on both Al-chelate resin affinity and HPLC columns.

Sequencing of the 64 min peak revealed a single amino acid sequence over the first 14 cycles with no other residues detected in significant amount. The results are shown in Table 1. The same sequence was obtained from both Vi- and BeF_x-trapped samples, once with Vi and two times with BeF_x. The sequence obtained is –FNEVVFNxPTRPDI–, corresponding to residues 1034 to 1047 of hamster isoform Pgp1 [22], which is known to be the Pgp isoform present in CR1R12 plasma membranes [9], residue 1033 of Pgp1 is Lys, consistent with trypsin cleavage, and the missing residue 'x' in the sequence corresponds to Tyr-1041. It is well established that 8-azido-ADP commonly labels Tyr in nucleotide binding sites, and the data show that Tyr-1041 is a primary target residue for 8-azido-ADP in the C-terminal NBS of Pgp.

Sequencing of the 51 min peak in each of three different experiments (comprising Vi- and BeF_x-trapped samples) showed that in no case it was homogeneous. How-

Table 1
Sequence of the radioactive peak eluting at 64 min from the HPLC column

Cycle	Vi-trapped 8-azido-ADP		BeF_x-trapped 8-azido-ADP	
	(aa)	(pmol)[a]	(aa)	(pmol)[a]
1	Phe	302.6	Phe	102.8
2	Asn	212.9	Asn	138.8
3	Glu	176.3	Glu	132.3
4	Val	213.6	Val	125.1
5	Val	221.5	Val	87.3
6	Phe	231.0	Phe	84.3
7	Asn	117.8	Asn	83.8
8	Xaa		Xaa	
9	Pro	64.2	Pro	28.0
10	Thr	29.9	Thr	15.5
11	Arg	19.1	Arg	23.2
12	Pro	26.9	Pro	22.3
13	Asp	36.7	Asp	65.0
14	Ile	103.7	Ile	85.5

[a]Yield of phenylthiohydantoin amino acid (aa) derivatives. In cycle 8 (X) no amino acid derivative was detected in significant amount, an unusual peak eluted just before the histidine derivative.

ever the major peptide sequence detected in all cases was –NIHFSxPSR–, corresponding to residues 393–401 of hamster isoform Pgp1. Residue 392 is Lys, consistent with trypsin cleavage, and the missing residue 'x' corresponds to Tyr-398. When the sequences of the N- and C-terminal nucleotide sites of hamster Pgp1 are aligned [22] it is seen that residue Tyr-398 exactly corresponds to residue Tyr-1041. Since it is established that after Vi- or BeF_x-trapping of 8-azido-ADP, followed by UV-illumination, both NBS of Pgp become labelled [16,17], we feel confident in stating that residue Tyr-398 is also a target residue for labelling of Pgp by 8-azido-ADP.

The data indicated that other sites of labelling by 8-azido-ADP occurred, and this is not unreasonable considering that the NBS of Pgp are known to be conformationally flexible [7]. Further fractionation of the 'flow-through' peak seen in Fig. 2 on a C18 HPLC column revealed several radioactive peaks, but in low yield. Moreover, sequencing revealed that they were still heterogeneous. Larger amounts of material than are currently available will be required to follow up this aspect.

Several labelling experiments were performed with purified Pgp, but were not successful. Our preparation of purified Pgp [10] contains a high lipid/protein ratio, and we ascribe our experimental difficulties to this fact. Future work will be aimed at obtaining active, pure Pgp preparations at low lipid/protein ratio.

4. Discussion

We demonstrate that 8-azido-ADP covalently labels residue Tyr-1041 of hamster Pgp isoform 1, contained within the sequence -FNEVVFNYPTRPDI- corresponding to residues 1034–1047. The same Tyr is present, within very similar adjacent sequences, in hamster Pgp2 and Pgp3, in human mdr1 and mdr2, and in all three Pgp isoforms in the mouse. It lies 32 residues upstream of the Lys of the Walker A consensus sequence, in the second (C-terminal) NBS of Pgp. Our results show that Tyr-1041 is a primary target residue of 8-azido-ADP and indicate that it lies close to the adenine ring of bound substrate MgATP.

In the past, the C-terminal NBS of Pgp has usually been predicted to start at a later point in the sequence than residue 1041. It may be recalled, however, that predictions of the starting position of the catalytic nucleotide binding domain in F_1-ATPase β-subunit [23] required revision after the X-ray structure [24] revealed that the nucleotide binding domain actually started well upstream from the predicted position.

Both Pgp nucleotide binding sites contain Walker A and B consensus sequences, which were originally discovered from studies of F_1-ATPase [25]. Thus F_1-ATPase may provide a useful analogy for Pgp. In F_1-ATPase catalytic sites a Tyr residue (βTyr-331) is stacked against, and makes Van der Waals contact with, the adenine ring of bound nucleotide [11], thereby playing an important role in binding and orienting the substrate. A similar role might exist for Tyr-1041 in Pgp. Substitution of F_1-ATPase βTyr-331 by Trp provided a valuable direct optical probe of nucleotide binding [11] and it will be interesting to determine whether the mutation Y1041W in Pgp can provide a similar, specific optical probe in the C-terminal nucleotide site of Pgp. It may also be mentioned that in F_1-ATPase, aromatic and hydrophobic residues surround the base moiety of bound substrate [24], and that the Pgp peptide sequence found above contains several such residues.

Our data indicated that residue Tyr-398 of Pgp is also labelled by 8-azido-ADP because the sequence -NIHFSxPSR- corresponding to Pgp residues 393–401 (where 'x' = Tyr-398), was the major sequence present in three different experiments in the labelled fraction eluting at 51 min in Fig. 2. When the sequences of the two Pgp nucleotide binding sites are aligned it is seen that Tyr-398 in the N-terminal NBS is equivalent to Tyr-1041 in the C-terminal NBS and like the latter, it lies 32 residues upstream of the Walker A consensus sequence Lys residue. The discussion presented above regarding residue Tyr-1041 therefore applies equally to residue Tyr-398.

In conclusion, this work has identified two Tyr residues, one in each NBS of Pgp, which lie close to the adenine ring of bound substrate MgATP and are very good candidates for Trp substitution and mutagenic analysis.

Acknowledgements: This work was supported by NIH grant GM50156.

References

[1] Gottesman, M.M. and Pastan, I. (1993) Annu. Rev. Biochem. 62, 385–427.
[2] Gros, P. and Buschman, E. (1993) Int. Rev. Cytol. 137C, 169–197.
[3] Shapiro, A.B. and Ling, V. (1995) J. Bioenerg. Biomembr. 27, 7–13.
[4] Germann, U.A. (1996) Eur. J. Cancer 32A, 927–944.
[5] Stein, W.D. (1997) Physiol. Rev. 77, 545–590.
[6] Senior, A.E., Al-Shawi, M.K. and Urbatsch, I.L. (1995) FEBS Lett. 377, 285–289.
[7] Senior, A.E., Al-Shawi, M.K. and Urbatsch, I.L. (1995) J. Bioenerg. Biomembr. 27, 31–36.
[8] Sharom, F.J., Yu, X., Chu, H.W.K. and Doige, C.A. (1995) Biochem. J. 308, 381–390.
[9] Al-Shawi, M.K., Urbatsch, I.L. and Senior, A.E. (1994) J. Biol. Chem. 269, 8986–8992.
[10] Urbatsch, I.L., Al-Shawi, M.K. and Senior, A.E. (1994) Biochemistry 33, 7069–7076.
[11] Weber, J. and Senior, A.E. (1997) Biochim. Biophys. Acta 1319, 19–58.
[12] Cornwell, M.M., Tsuruo, T., Gottesman, M.M. and Pastan, I. (1987) FASEB J. 1, 51–54.

[13] Azzaria, M., Schurr, E. and Gros, P. (1989) Mol. Cell. Biol. 9, 5289–5297.
[14] Georges, E., Zhang, J.-T. and Ling, V. (1991) J. Cell. Physiol. 148, 479–484.
[15] Urbatsch, I.L., Sankaran, B., Weber, J. and Senior, A.E. (1995) J. Biol. Chem. 270, 19383–19390.
[16] Urbatsch, I.L., Sankaran, B., Bhagat, S. and Senior, A.E. (1995) J. Biol. Chem. 270, 26956–26961.
[17] Sankaran, B., Bhagat, S. and Senior, A.E. (1997) Biochemistry 36, 6847–6853.
[18] Sankaran, B., Bhagat, S. and Senior, A.E. (1997) Arch. Biochem. Biophys. 341, 160–169.
[19] Al-Shawi, M.K. and Senior, A.E. (1993) J. Biol. Chem. 268, 4197–4206.
[20] Shoemaker, M.T. and Haley, B.E. (1993) Biochemistry 32, 1883–1890.
[21] Sankaran, B., Chavan, A.J. and Haley, B.E. (1996) Biochemistry 35, 13501–13510.
[22] Endicott, J.A., Sarangi, F. and Ling, V. (1991) DNA Sequence 2, 89–101.
[23] Duncan, T.M., Parsonage, D. and Senior, A.E. (1986) FEBS Lett. 208, 1–6.
[24] Abrahams, J.P., Leslie, A.G.W., Lutter, R. and Walker, J.E. (1994) Nature 370, 621–628.
[25] Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J. (1982) EMBO J. 1, 945–951.