FEBS Letters 458 (1999) 292–294 FEBS 22612

NEM modification prevents high-affinity ATP binding to the first nucleotide binding fold of the sulphonylurea receptor, SUR1

Michinori Matsuo[a], Stephen J. Tucker[b], Frances M. Ashcroft[b], Teruo Amachi[a], Kazumitsu Ueda[a,*]

[a] *Laboratory of Biochemistry, Division of Applied Life Sciences, Kyoto University Graduate School of Agriculture, Kyoto 606-8502, Japan*
[b] *University Laboratory of Physiology, Oxford OX1 3PT, UK*

Received 7 July 1999; received in revised form 11 August 1999

Abstract Pancreatic β-cell ATP-sensitive potassium channels, composed of SUR1 and Kir6.2 subunits, serve as a sensor for intracellular nucleotides and regulate glucose-induced insulin secretion. To learn more about the interaction of SUR1 with nucleotides, we examined the effect of *N*-ethylmaleimide (NEM) modification. Photoaffinity labeling of SUR1 with 5 μM 8-azido-[α-^{32}P]ATP or 8-azido-[γ-^{32}P]ATP was inhibited by NEM with K_i of 1.8 μM and 2.4 μM, and Hill coefficients of 0.94 and 1.1, respectively. However, when the cysteine residue in the Walker A motif of the first nucleotide binding fold (NBF1) of SUR1 was replaced with serine (C717S), photoaffinity labeling was not inhibited by 100 μM NEM. These results suggest that NBF1 of SUR1 has a NEM-sensitive structure similar to that of NBF1 of MDR1, a multidrug transporter, and confirm NBF1 as the high-affinity ATP binding site on SUR1.

Key words: *N*-Ethylmaleimide; ABC transporter; ATP binding; Potassium channel

1. Introduction

ATP-sensitive potassium (K_{ATP}) channels of pancreatic β-cells regulate insulin release by modulating the β-cell membrane potential [1–4]. Because these channels are inhibited by ATP, and activated by MgADP, they are thought to act as a sensor for intracellular nucleotides. However, it is not yet clear how they monitor changes in the concentrations of intracellular ATP and ADP. The K_{ATP} channel is a heterooctamer composed of four sulphonylurea receptor (SUR1) and four Kir6.2 subunits [5–8]. Like P-glycoprotein (MDR1), the multidrug resistance-related protein (MRP1), and the cystic fibrosis transmembrane conductance regulator (CFTR), SUR1 is a member of the ATP binding cassette (ABC) superfamily [9,10]. These proteins have two nucleotide binding folds (NBFs). Recently, we reported that SUR1 is efficiently photoaffinity labeled with 8-azido-[^{32}P]ATP even in the absence of Mg^{2+} and that unlike MDR1, high-affinity labelling occurs preferentially at a single NBF [11,12]. These features of ATP binding by SUR1 are different from that of MDR1, in which both NBFs can be labeled in an orthovanadate- and Mg^{2+}-dependent manner [13,14].

Chemical modification of specific amino acids provides a means of analyzing structure-function relationships. In particular, cysteine residues are useful targets for chemical modification because of their specific and covalent modification by many different thiol-modifying agents. Effects of the thiol-modifying agent *N*-ethylmaleimide (NEM) have been well studied on the ATP binding and hydrolysis properties of MDR1. Although human MDR1 contains seven cysteines, only two of them, located in the Walker A motifs of the NBFs, can be readily modified by NEM [14,15]. Covalent modification of a single cysteine in the Walker A motif of either NBF is sufficient to inactivate ATPase activity [15] or vanadate-induced nucleotide trapping of MDR1 [14]. However, the effects of NEM modification of the cysteine in NBF1 and NBF2 on 8-azido-ATP binding to MDR1 are not equivalent. NEM modification of the cysteine in NBF2 did not appear to affect 8-azido-ATP binding to MDR1, whereas NEM modification of the cysteine in NBF1 significantly reduced it [14]. Like MDR1, the Walker A motif of NBF1 of SUR1 contains a cysteine residue. We therefore examined the effect of NEM on 8-azido-ATP binding to SUR1. We show here that NEM inhibits ATP binding to NBF1 of SUR1, as it does for MDR1 [14], despite the fact that several other properties of ATP binding differ for these two ABC transporters.

2. Materials and methods

2.1. Materials

8-Azido-[α-^{32}P]ATP and 8-azido-[γ-^{32}P]ATP were purchased from ICN Biomedicals. Hamster SUR1 (K719M) cDNA was generously provided by Dr Susumu Seino (Chiba University, Japan). Wild-type rat SUR1 cDNA was the kind gift of Dr G. Bell (University of Chicago, USA). The C717S and K1385M mutations used in this study were made in rat SUR1. Hamster SUR1 and rat SUR1 exhibit identical ATP binding properties (data not shown).

2.2. Covalent modification of SUR1 with NEM

Membranes from COS-7 cells expressing SUR1, prepared as described [11], were incubated with NEM in TE buffer (40 mM Tris-HCl (pH 7.5), 0.1 mM EGTA) for 10 min at 25°C and washed twice with TE buffer containing 50 mM dithiothreitol.

2.3. Photoaffinity labeling with 8-azido-[^{32}P]ATP

Membranes treated with NEM were incubated with 5 μM 8-azido-[α-^{32}P]ATP or 8-azido-[γ-^{32}P]ATP in 10 μl TEM buffer (40 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, 1 mM $MgSO_4$) containing 2 mM ouabain for 10 min at 37°C. The reactions were stopped by the addition of 500 μl of ice-cold TEM buffer, and free 8-azido-[^{32}P]ATP was removed after centrifugation (15000×*g*, 5 min, 2°C). Pellets were resuspended in 8 μl of TEM buffer and irradiated for 5 min (at 254 nm, 5.5 mW/cm^2) on ice. Samples were electrophoresed on a 7% SDS-polyacrylamide gel, and autoradiographed. The 8-azido-[α-^{32}P]ATP-labeled SUR1 was measured by scanning with a radioimaging analyser (BAS2000, Fuji Photo Film Co.). Experiments were done in triplicate.

*Corresponding author. Fax: (81) (75) 753-6104.
E-mail: uedak@kais.kyoto-u.ac.jp

PII: S0014-5793(99)01170-9

3. Results and discussion

Covalent modification with thiol-modifying agents prevents ATP hydrolysis and ATP binding by the ABC-superfamily proteins MDR1 [14–17] and CFTR [18]. To learn more about the interaction of SUR1 with nucleotides, we examined the effect of covalent modification by NEM on ATP binding to SUR1.

Membranes from COS-7 cells transiently expressing SUR1 were incubated with 5 μM 8-azido-[α-^{32}P]ATP or 8-azido-[γ-^{32}P]ATP. After removal of the unbound ligands, the proteins were irradiated with UV light. SUR1 was efficiently photoaffinity labeled by both ligands (Fig. 1). When membranes were pretreated with NEM, photoaffinity labeling of SUR1 by either ligand was inhibited by NEM in a concentration-dependent manner (Fig. 1A). The data were fit by the Hill equation with K_i values of 1.8 μM and 2.4 μM, and Hill coefficients of 0.94 and 1.1, for binding of 8-azido-[α-^{32}P]ATP and 8-azido-[γ-^{32}P]ATP, respectively (Fig. 1B). Similar results were obtained when the reaction was carried out in the absence of Mg^{2+} (data not shown). These results confirm that the labeling observed is due to binding of ATP rather than phosphorylation. The Hill coefficient of unity further suggests that modification of a single site by NEM is sufficient to prevent ATP binding.

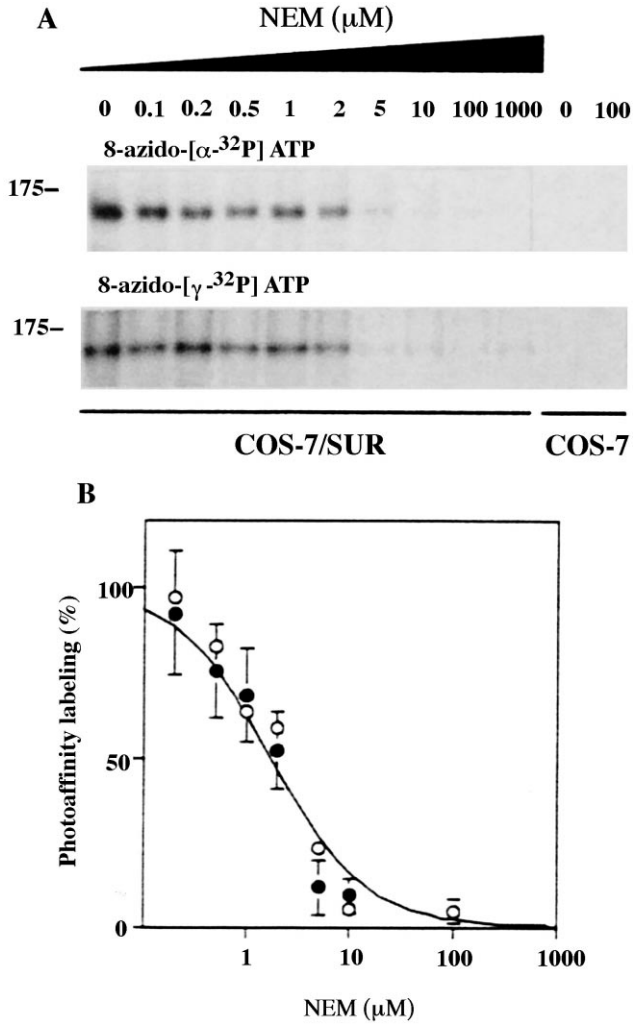

Fig. 1. Inhibition of high-affinity 8-azido-ATP labeling of SUR1 by NEM. A: Membrane proteins (20 μg) from COS-7 cells expressing SUR1 were treated with the indicated concentrations of NEM and incubated with 5 μM 8-azido-[α-^{32}P] or 8-azido-[γ-^{32}P] ATP at 37°C for 10 min. Proteins were photoaffinity labeled as described in Section 2. B: Relative photoaffinity labeling of SUR1 after NEM treatment with 8-azido-[α-^{32}P] ATP (○) or 8-azido-[γ-^{32}P]ATP (●), expressed as a percentage of that obtained for NEM-untreated SUR1. The data were fit using the Hill equation. Experiments were done in triplicate.

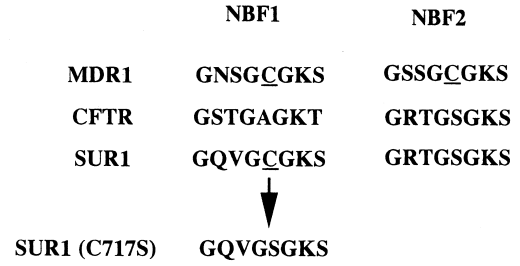

Fig. 2. Comparison of the Walker A sequences of MDR1, CFTR, and SUR1. The cysteine residue within the Walker A motif of NBF1 of SUR1 was replaced with serine in the C717S mutant form.

Fig. 2 compares the amino acid sequences of the Walker A motifs of NBF1 and NBF2 of SUR1, MDR1 and CFTR. A cysteine residue is present in both NBFs of MDR1 but neither NBF of CFTR. NBF1 of SUR1 contains a cysteine residue at the analogous site to that in NBF1 of MDR1. It has been reported previously that modification of the Walker A cysteine residue in NBF1 is responsible for the inhibition of ATP binding to MDR1 by NEM [14], and that modification of a cysteine engineered into the Walker A motif of either NBF of CFTR partially inhibited Cl^- channel activity, possibly via decreased ATP binding [19]. This suggests that the cysteine residue within the Walker A motif of NBF1 is the site of NEM modification of ATP binding to SUR1. We therefore examined the effects of NEM on 8-azido-ATP binding to a mutant form of SUR1, in which the cysteine residue within the Walker A motif of NBF1 was replaced with serine (C717S).

Membranes containing equal amounts of the wild-type and the C717S mutant form of SUR1 were treated with 100 μM NEM, and photoaffinity labeling with 5 μM 8-azido-[γ-^{32}P]-ATP was then examined (Fig. 3). Both wild-type and C717S SUR1 were photoaffinity labeled to the same extent in the absence of NEM. However, by contrast to wild-type SUR1, photoaffinity labeling of C717S SUR1 was unaffected by pretreatment with NEM. Like wild-type SUR1, the K1385M mutant form of SUR1 (in which the lysine residue in the Walker A motif of NBF2 was replaced with methionine) was photoaffinity labeled by 8-azido-ATP and this labeling was inhibited by NEM treatment. However, the K719M mutant form of SUR1 (in which the lysine residue within the Walker A motif of NBF1 was replaced with methionine) was not photoaffinity labeled either in the absence or in the presence of NEM. These results indicate that cysteine-717 within NBF1 of SUR1 is responsible for inhibition of high-affinity 8-azido-ATP binding by NEM, and suggest that NBF1 of SUR1 has a NEM-sensitive structure similar to that of NBF1 of MDR1.

We have reported previously that mutations in either the Walker A or B motifs of NBF1, K719M and D854N, abolish

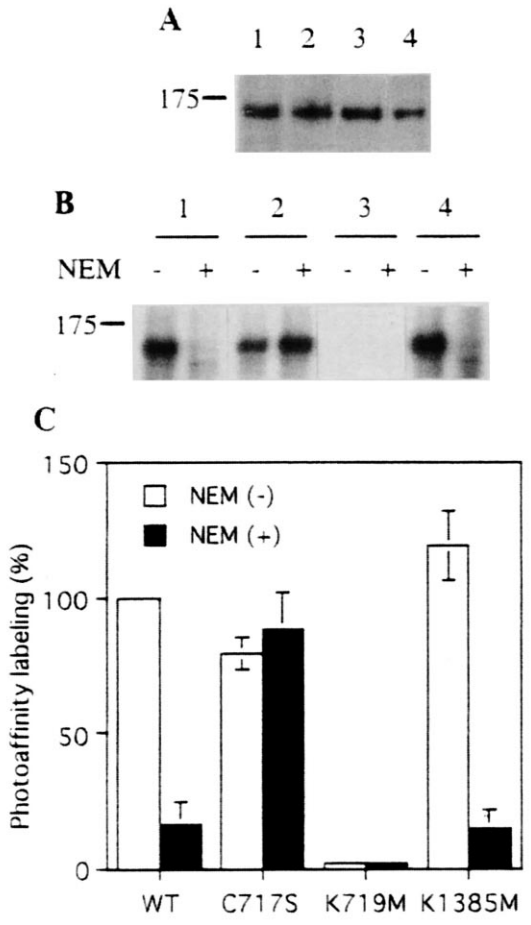

Fig. 3. Effect of NEM on photoaffinity labeling of SUR1 mutants. A: Immunoblotting of membrane proteins (7–20 μg) from COS-7 cells expressing wild-type (lane 1), C717S (lane 2), K719M (lane 3), or K1385M (lane 4) mutant forms of SUR1 using an antibody against the C-terminal 21 amino acids of rat SUR1. B: Membranes containing equivalent amounts of wild-type and mutant forms of SUR1 were treated with or without 100 μM NEM, and photoaffinity labeled. C: Relative photoaffinity labeling of SUR1 expressed as a percentage of that of NEM-untreated wild-type SUR1. White bars, NEM-untreated; black bars, NEM-treated. Experiments were done in triplicate.

high-affinity 8-azido-ATP binding to SUR1, whereas equivalent mutations in NBF2 do not affect ATP binding [11]. Also we have reported that MgADP and MgATP stabilised binding of prebound 8-azido-ATP to SUR1, while mutations in the Walker A and B motifs of NBF2 abolished this stabilising effect of MgADP [12]. These results suggested that SUR1 binds 8-azido-ATP strongly at NBF1, and MgADP at NBF2, and that the two NBFs cooperate in nucleotide binding. The inhibition of this high-affinity 8-azido-ATP binding to SUR1 by NEM and the lack of inhibition found with the C717S mutation now confirms that NBF1 is the high-affinity ATP binding site identified on SUR1.

It has been reported that thiol-modifying agents, including NEM and *p*-chloromercuriphenylsulphonate (pCMPS), inhibit both β-cell and cardiac muscle K_{ATP} channels irreversibly and that the presence of ATP protects against this effect [20–22]. However, the inhibitory effect of pCMPS on the β-cell K_{ATP} channel is due to interaction of this thiol-modifying agent with the cysteine at position 42 of Kir6.2 [23] and not C717 of SUR1. Unlike NEM, pCMPS does not inhibit high-affinity ATP binding to SUR1 (data not shown). This implies that, in comparison to NEM, the bulkier and more hydrophobic pCMPS reagent probably cannot access C717 of SUR1 in the Kir6.2/SUR1 channel complex. Therefore, it might be worth examining whether the ability of MgATP to activate Kir6.2/SUR1 currents [24], which is mediated by the NBFs of SUR1, can be influenced by NEM modification.

In summary, NBF1 of SUR1 has a NEM-sensitive structure similar to NBF1 of MDR1, although their ATP binding properties appear quite different. Our study adds further support for the idea that the NBFs of SUR1 and MDR1 differ in their interaction with ATP and provides strong evidence that the high-affinity ATP binding site on SUR1 resides in the first nucleotide binding fold.

Acknowledgements: We thank Dr S. Seino (Chiba University, Japan) for providing hamster SUR1 (K719M) cDNA and the antibody against SUR1. This work was supported by Grants-in-Aid for Scientific Research on Priority Areas 'ABC proteins' from the Ministry of Education, Science, Sports, and Culture of Japan, by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists and by the Wellcome Trust.

References

[1] Nichols, C.G., Shyng, S.-L., Nestorowicz, A., Glaser, B., Clement IV, J.P., Gonzalez, G., Aguilar-Bryan, L., Permutt, M.A. and Bryan, J. (1996) Science 272, 1785–1787.
[2] Ashcroft, F.M. and Gribble, F.M. (1998) Trends Neurosci. 21, 288–294.
[3] Aguilar-Bryan, L., Clement IV, J.P., Gonzalez, G., Kunjilwar, K., Babenko, A. and Bryan, J. (1998) Physiol. Rev. 78, 227–245.
[4] Seino, S. (1999) Annu. Rev. Physiol. 61, 337–362.
[5] Inagaki, N., Gonoi, T. and Seino, S. (1997) FEBS Lett. 409, 232–236.
[6] Shyng, S.-L. and Nichols, C.G. (1997) J. Gen. Physiol. 110, 655–664.
[7] Clement IV, J.P., Kunjilwar, K., Gonzalez, G., Schwanstecher, M., Panten, U., Aguliar-Bryan, L. and Bryan, J. (1997) Neuron 18, 827–838.
[8] Zerangue, N., Schwappach, B., Jan, Y.N. and Jan, L.Y. (1999) Neuron 22, 537–548.
[9] Higgins, C.F. (1992) Annu. Rev. Cell Biol. 8, 67–113.
[10] Aguilar-Bryan, L., Nichols, C.G., Wechsler, S.W., Clement IV, J.P., Boyd III, A.E., González, G., Herrera-Sosa, H., Nguy, K., Bryan, J. and Nelson, D.A. (1995) Science 268, 423–426.
[11] Ueda, K., Inagaki, N. and Seino, S. (1997) J. Biol. Chem. 272, 22983–22986.
[12] Ueda, K., Komine, J., Matsuo, M., Seino, S. and Amachi, T. (1999) Proc. Natl. Acad. Sci. USA 96, 1268–1272.
[13] Urbatsch, I.L., Sankaran, B., Bhagat, S. and Senior, A.E. (1995) J. Biol. Chem. 270, 26956–26961.
[14] Takada, Y., Yamada, K., Taguchi, Y., Kino, K., Matsuo, M., Tucker, S.J., Komano, T., Amachi, T. and Ueda, K. (1998) Biochim. Biophys. Acta 1373, 131–136.
[15] Loo, T.W. and Clarke, D.M. (1995) J. Biol. Chem. 270, 22957–22961.
[16] Al-Shawi, M.K., Urbatsch, I.L. and Senior, A.E. (1994) J. Biol. Chem. 269, 8986–8992.
[17] Liu, R. and Sharom, F.J. (1996) Biochemistry 35, 11865–11873.
[18] Szabó, K., Szakacs, G., Hegedus, T. and Sarkadi, B. (1999) J. Biol. Chem. 274, 12209–12212.
[19] Cotten, J.F. and Welsh, M.J. (1998) J. Biol. Chem. 273, 31873–31879.
[20] Weik, R. and Neumcke, B. (1989) J. Membr. Biol. 110, 217–226.
[21] Lee, K., Ozanne, S.E., Hales, C.N. and Ashford, M.L.J. (1994) J. Membr. Biol. 139, 167–181.
[22] Coetzee, W.A., Nakamura, T.Y. and Faivre, J.-F. (1995) Am. J. Physiol. 269, 1625–1633.
[23] Trapp, S., Tucker, S.J. and Ashcroft, F.M. (1998) J. Gen. Physiol. 112, 325–332.
[24] Gribble, F.M., Tucker, S.J., Haug, T. and Ashcroft, F.M. (1998) Proc. Natl. Acad. Sci. USA. 95, 7185–7190.