Minireview

"Lysine is the Lord", thought some scientists in regard to the group interacting with fluorescein isothiocyanate in ATP-binding sites of P-type ATPases But, is it not cysteine?

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Abstract. Isothiocyanates are recognized inhibitors acting on ATP-binding sites of P-type ATPases. Detailed studies with modification of proteins in molecules of purified ATPases by fluorescein isothiocyanate (FITC) and consequent tryptic hydrolysis followed by isolation and sequencing of the respective peptide fragments revealed FITC bound to a lysine residue. This residue was then indicated to be essential for the interaction of ATP with the P-type ATPases. Nevertheless, upon an exchange by site directed mutagenesis of lysine, believed to be essential, the expected total inhibition of ATPase activity was missing. In addition, in the case of the plasma membrane Ca^{2+}-ATPase, the residual activity still remained sensitive to FITC. It was attempted to explain the latter finding by hypothetical existence of some other lysine residue essential for the ATPase activity. On the contrary, in our previous studies we have shown that, based on the reactivity of isothiocyanates, the primary target of FITC in P-type ATPases has to be the SH group of a cysteine residue. However, later on, in altered conditions during trypsinolysis and sequencing, FITC may become transferred from its original site of interaction to a lysine residue and this may lead to final identification of the label on a false place. The present study represents an attempt of elucidating the controversy whether it is lysine or cysteine that represents the FITC-sensitive group truly responsible for the recognition by the active site of P-type ATPases of ATP and its binding.

Key words: P-type ATPases — ATP dependent proteases — ATP-binding site — Isothiocyanates — Thiol and amino groups

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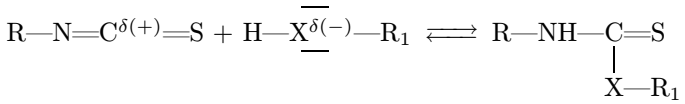
Affinity versus stability in reactions of isothiocyanates

High reactivity of isothiocyanates (ITC) with nucleophilic compounds (NC) is based on a partial lack of electron density localized on the carbon atom in the —NCS group. The reaction itself may be represnted by the simplified equation:



In proteins, the —SH, —NH_2 and —OH groups of cysteine, lysine and serine residues represent adequate partners for the interaction with isothiocyanates. When expressing the affinity of a primary aminogroup for reaction with ITC in arbitrary units (and setting it equal to 1), then the affinity of the hydroxyl group will be 0.2 and the affinity of the sulfhydryl group will range within the interval 1,000—1,000,000 (Drobnica and Gemeiner 1976; Drobnica et al. 1977; Gemeiner and Drobnica 1979). This means that upon reacting with proteins, ITC will choose —SH with highest preference. Nevertheless, the resulting reaction products S-esters of dithiocarbamic acid exhibit low thermodynamic stability, i.e. they dissociate easily demonstrating the reversible character of this reaction. The reaction of ITC with amino groups runs much slowlier and provides considerably more stable N, N′-disubstituted derivatives of thiourea. Moreover, the latter reaction seems to be almost irreversible (Drobnica and Gemeiner 1976; Drobnica et al. 1977; Gemeiner and Drobnica 1979).

In the process of isolation of ITC-labeled proteins that follows, the excess of ITC, which does not participate in the reaction, becomes removed. Consequently, because of its low equilibrium constant the primarily formed ITC—SH reaction product undergoes a fast decay. The ITC liberated may enter further reactions with —SH, —NH_2 or —OH groups with diverse preferences but, eventually only the product with —NH_2 groups remains stable and this leads to its gradual accumulation (Wilderspin and Green 1983; Swoboda and Hasselbach 1985).

Preferential interaction of isothiocyanates with SH-groups of membrane proteins could be documented by the finding of Santos et al. (1999). These authors showed that sulfhydryl groups of cysteine residues are modified by 4,4'-diisothiocyanatostilbene-2, 2'-disulfonic acid when this substance was applied to modulate pNPPase activity of the plasma membrane calcium pump.

Reaction of ITC with P-type ATPases

P-type ATPases represent well-known systems for active transports of cations, but they are still extensively studied (Nakamura et al. 1997; Breier et al. 1998; Obšil et al. 1998; Sulová et al. 1998; Tsuda et al. 1998; Ward and Cavieres 1998; Gatto

Table 1. Amino acid sequence of oligopeptides that represent FITC sensitive ATP binding site of P-type ATPases.

ATPase	Sequence			
(H^+/K^+)-ATPase 1	**K**GA	PER	VLE	R***C***S
(H^+/K^+)-ATPase 2	**K**GA	PER	ILE	K***C***S
(Na^+/K^+)-ATPase	**K**GA	PER	ILD	R***C***S
$SRCa^{2+}$-ATPase	**K**GA	PEG	VID	R***C***T
$PMCa^{2+}$-ATPase 1	**K**GA	SEI	ILK	K***C***F
$PMCa^{2+}$-ATPase2	**K**GA	SEI	VLK	K***C***C

(H^+/K^+)-ATPase 1 – from gastric mucosa (Shull and Lingrel 1986); (H^+/K^+)-ATPase 2 – hypothetical enzyme from distal colon (Crowson and Shull 1992); (Na^+/K^+)-ATPase (Shull et al. 1985); $SRCa^{2+}$-ATPase (MacLennan et al. 1985); identical structures have been described for all isoenzymes SERCA1, SERCA2a, SERCA2b and SERCA3 (Inesi and Kirtley 1992); $PMCa^{2+}$-ATPase (1) – plasmalemmal Ca^{2+}-ATPase isoenzyme 1 (Verma et al.1988); $PMCa^{2+}$-ATPase (2) – plasmalemmal Ca^{2+}-ATPase isoenzyme 2 (Shull and Greeb 1988).

et al. 1999; Scheiner-Bobis and Schreiber 1999; Vrbjar et al. 1999; Lambrecht et al. 2000; Ziegelhöffer et al. 2000).

FITC has been often applied to label the ATP-binding site of P-type ATPases (Carilli et al. 1981; Pick and Bassilian 1981; Mitchinson et al. 1982; Farley et al. 1984; Kirley et al. 1984; Farley and Faller 1985; Ohta et al. 1985; Filoteo et al. 1987; Lin and Faller 1993; Smirnova and Faller 1993; Nakamura et al. 1997; Tsuda et al. 1998; Ward and Cavieres 1998). After being cleaved by trypsin the ITC label bearing oligopeptides were purified and sequenced (Mitchinson et al. 1982; Farley et al. 1984; Kirley et al. 1984; Farley and Faller 1985; Ohta et al. 1985; Filoteo et al. 1987). These oligopeptides exhibited considerable homology in their sequence. Five from eleven amino acids were identical in all P-type ATPases from animal tissue according to the formula K, G, A, X, E, X, V/I, L/I, X, R/K, C (Table 1). The label was always found to be linked with a lysine first in the sequence. Based on these findings it was concluded that the lysine bearing the label is essential for binding of ATP and/or ATPase activity of these enzymes. On the other hand, we could demonstrate that the activity of (Na^+/K^+)-ATPase may also be competitively inhibited by isothiocyanates that are bound exclusively to the —SH group of the cysteine residue (Ziegelhöffer et al. 1983, 1987, 2000; Breier et al. 1989, 1995, 1996). The same studies also revealed that, in spite of the difference in the site of interaction, all features of inhibition seen after the interaction of ITC with cysteine were similar to those described when the ITC was found to be localized on lysine. Based on this finding and the knowledge about the reactivity of ITC, we concluded that cysteine rather than lysine represents the group which first recognizes and binds ATP in the active site of the (Na^+/K^+)-ATPase. Our conclusion became strongly supported by experiments on various P-type ATPases

such as the (Na^+/K^+)-, $SRCa^{2+}$- and $PMCa^{2+}$-ATPase in which lysine believed to be essential for ITC binding was replaced with other amino acids by means of site directed mutagenesis (Maruyama and MacLennan 1988; Wang and Farley 1992; Adamo et al. 1996). Namely, these studies did not result in the expected complete loss of activities of the respective enzymes, even the opposite: the residual activity of mutant $PMCa^{2+}$ ATPase continued to be FITC sensitive (Adamo et al. 1996). The explanation for the latter finding by hypothesising the existence of another lysine that, in the absence of the first "essential one", will replace it in its function (Adamo et al. 1996) may hardly be considered as plausible. This is because no second labeled lysine could be demonstrated in studies analyzing tryptic fragments after a complete inhibition of ATPase by ITC. Hence, it seems much more probable that FITC inhibits the ATPases by modification of cysteine which, because of the described instability of the reaction product, cannot be finally detected as the original site of the ITC interaction.

The role of the SH groups in ATP binding can be documented by the finding of Scheiner-Bobis et al. (1992) that sulfhydryl reactive ATP analogue (8-thiocyanato-ATP) was shown: i) to inhibit the activity of (Na^+/K^+)-ATPase and kinases and ii) to interact exclusively with the ATP binding domain of this enzymes. Gatto et al. (1999) have recently identified Cys577 as a "*conformationally mobile residue in the ATP-binding domain of (Na^+/K^+)-ATPase α-subunit*". The importance of the SH-group of cysteine for the interaction with ATP in its specific binding sites may be also documented on P-glycoprotein (a transport ATPase of the plasma membrane with two similar ATP-binding sites exhibiting ABC consensus). This protein contains thiol groups essential for ATP binding in its both ATP-binding sites (al-Shawi et al. 1994; Liu and Sharom 1996, 1997) which exhibit the following structure: XSGCGKY, when X represents N or S and Y represents T or S (Van der Bliek et al. 1987, 1988; Lincke et al. 1991).

The way how ATP may bind in the ATP binding sites of the P-type ATPases

Our hypothesis about the mode of ATP binding in the ATP binding sites of P-type ATPases is based on the essential involvement of a cysteine SH-group and the partial importance of a lysine NH_2-group. The former one may recognize the ATP molecule and bind it by forming a hydrogen bond with the 6-amino-group on the adenine part of ATP (Patzelt-Wenczler and Schoner 1981). The NH_2-group of lysine may be important for accommodation of negatively charged phosphate groups of ATP (Breier et al. 1996; Scheiner-Bobis and Schreiber 1999; Ziegelhöffer et al. 2000). Such a role of lysine in ATP binding is also confirmed by the finding that its replacement by the negatively charged glutamic acid residue induces a dramatical diminution of the affinity of the (Na^+/K^+)-ATPase and $SRCa^{2+}$-ATPase to ATP (Maruyama and MacLennan 1988; Wang and Farley 1992). This lysine was recently assumed to arrest β- or (α-) phosphate of ATP in a proper position prior to hydrolysis of γ-phosphate group (Scheiner-Bobis and Schreiber 1999).

Table 2. Amino acid sequence of oligopeptides, that represent the ATP binding site of ATP dependent proteases.

Enzyme	Sequence			
Human mitochondria lon protease[1]	***C***FY	GPP	GVG	**K**
Mitochondria lon protease from S. *Cerevisiae*[2,3,4]	***C***FV	GPP	GVG	**K**
ATP-dependent protease LA1 from *B. Subtilis*[5]	***C***LA	GPP	GVG	**K**
ATP-dependent protease LA from *E. Coli*[6,7,8,9,10]	***C***FY	GPP	GVG	**K**

[1]Amerik et al. (1994), [2]Van Dyck et al. (1994), [3]Suzuki et al. (1994), [4]Hahn et al. (1988), [5]Riethdorf et al. (1994), [6]Thomas et al. (1993). [7]Amerik et al. (1988). [8]Fischer and Glockshuber (1993), [9]Chin et al. (1988).[10]Gayda et al. (1985).

The geometric arrangement of the ATP-binding site of the (Na^+/K^+)-ATPase and $PMCa^{2+}$-ATPase were investigated by using the Desktop Molecular Modeling software (Oxford Electronic Publishing, UK). This procedure revealed that there might really exist structures of the ATP binding oligopeptides (see Table 1) in which the distances between the atom of S in cysteine and the atom of N in the ε-amino-group of lysine are 1.235 nm and 1.228 nm for (Na^+/K^+)-ATPase and $PMCa^{2+}$-ATPase, respectively. These distances match with the distance of 1.097 nm or 1.010 nm between the atom of H of the amino-group in position 6 and the atom of O of the β- or α-phosphate group in the ATP molecule. The possibility of ATP binding oligopeptide to bind ATP in the manner described above was checked by the computation of the three-dimensional structure of complexes formed from ATP binding peptide (for (Na^+/K^+)-ATPase) and ATP (Fig. 1), using ACD/ChemSketch software (produce by Advanced Chemistry Development, Inc., Canada). This procedure revealed that the respective complexes might really exist in both cases, i.e., when ATP is interacting with lysine 480 by α or β phosphate group and with cysteine 490 by 6-amino group of adenine.

ATP-dependent proteases (lon proteases) may be considered a group of enzymes exhibiting partial similarities as compared with P-type ATPases in ATP binding sites. The structure of the ATP binding sites of ATP-dependent proteases from different species exhibits a considerable homology (Tab. 2) documenting the localization of both, a cysteine and a lysine residue that are divided by a span of 8 amino-acids (Gayda et al. 1985; Amerik et al. 1988; Chin et al. 1988; Hahn et al. 1988; Fischer and Glockshuber 1993; Thomas et al. 1993; Amerik et al. 1994; Suzuki et al. 1994; Van Dyck et al. 1994; Riethdorf et al. 1994). In this respect, the P-type ATPases differ from proteases in the length of the span 9 amino acids lons, as well as in the opposite orientation of the sequence starting in ATPases with the lysine containing part directed to the N terminal. Nevertheless, from the described structural arrangements of the ATP binding sites of P-type ATPases and ATP dependent proteases it may be assumed that both groups of enzymes will interact with the ATP molecule in a similar way.

Figure 1. Three dimensional structures of complexes formed by interaction of ATP binding oligopeptide from (Na^+/K^+)-ATPase (Table 1) and ATP. *Upper structure* – Example of 6-aminogroup of ATP interacting with —SH group of cysteine 490 and β-phosphate of ATP interacting with NH_2 group of lysine 480. *Lower structure* – Example of 6-aminogroup of ATP interacting with —SH group of cysteine 490 and α-phosphate of ATP interacting with NH_2 group of lysine 480. Undefined bonds between phosphates of ATP and NH_2 groups of oligopeptide as well as 6-aminogroup of ATP and – SH group of oligopeptide were chosen for the computation. The structures of the complexes were computed using ACD/ChemSketch software (produced by Advanced Chemistry Development, Inc. Canada). This software is limited to molecules that contain less than 200 atoms. To meet this condition, both argentines in this structure had to be replaced by glycines.

In conclusion, we could stress that cysteine in the ATP binding oligopeptide of P-type ATPases (Table 1) is probably essential for ATP binding, and moreover represents the probable target for attack by thiol reacting substances, that inhibit ATPase activity (Ziegelhöffer et al. 1983, 1987, 2000; Scheiner-Bobis et al. 1992; Breier et al 1995, 1996; Gatto et al. 1999; Lambrecht et al. 2000). Nevertheless, the possibility that other cysteine residues, for example cysteine 577 in (Na^+/K^+)-ATPase (Gatto et al. 1999), is essential from these aspects could not be excluded either.

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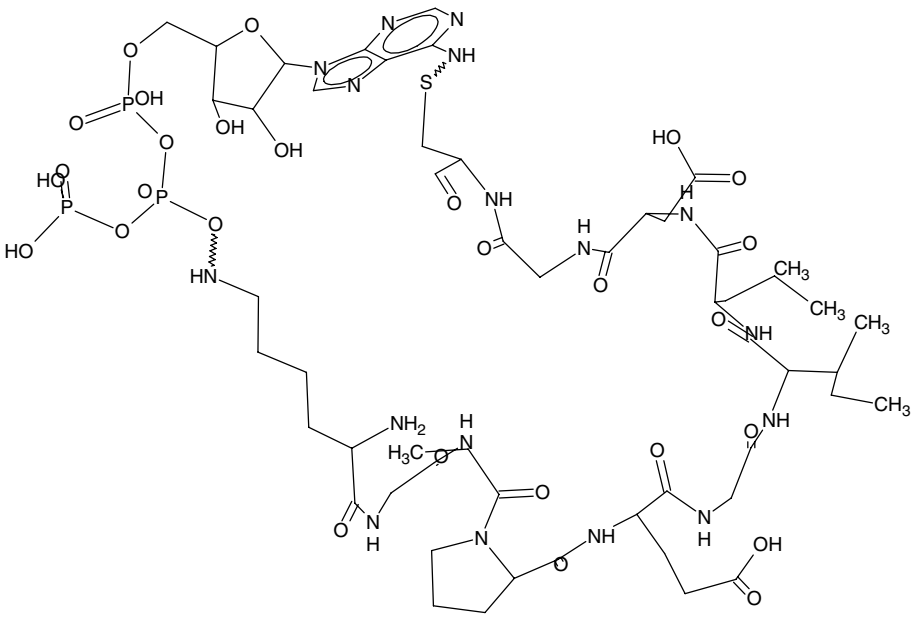

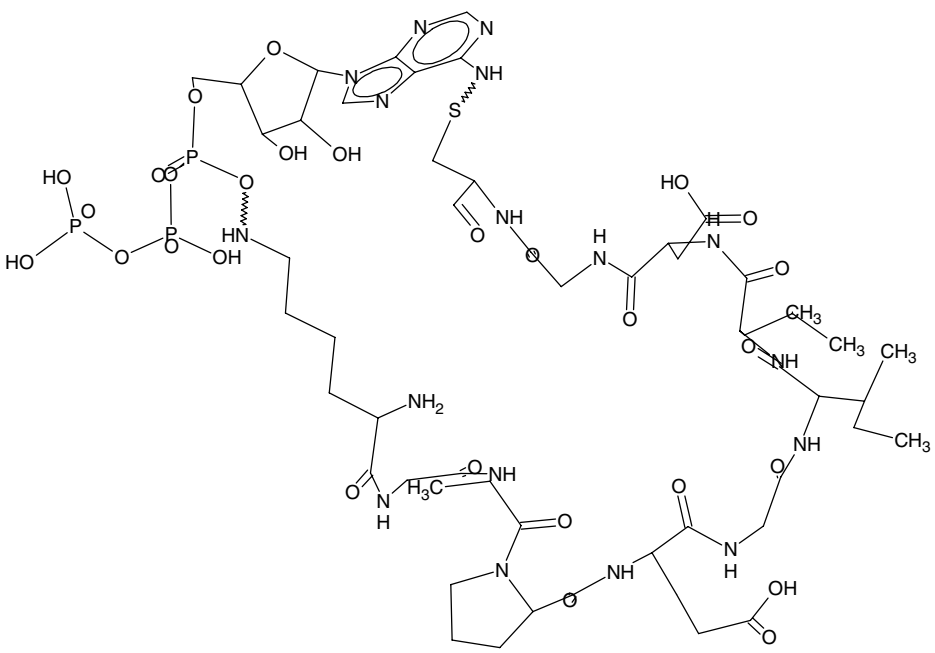

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