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# Modification of the Mitochondrial Sulfonylurea Receptor by Thiol Reagents

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**The purpose of this study was to investigate the effects exerted by thiol-modifying reagents on themitochondrial sulfonylurea receptor. The thiol-oxidizing agents (timerosal and 5,5′-dithio-bis(2-nitrobenzoic acid)) were found to produce a large inhibition (70% to 80%) of specific binding of [$^3$H]glibenclamide to the beef heart mitochondrial membrane. Similar effects were observed with membrane permeable (N-ethylmaleimide) and non-permeable (mersalyl) thiol modifying agents. Glibenclamide binding was also decreased by oxidizing agents (hydrogen peroxide) but not by reducing agents (reduced gluthatione, dithiothreitol and the 2,3-dihydroxy-1,4-dithiolbutane). The results suggest that intact thiol groups, facing the mitochondrial matrix, are essential for glibenclamide binding to the mitochondrial sulfonylurea receptor.**


***Key Words:*** **glibenclamide; mitochondrial sulfonylurea receptor; SH reagents; mitochondria.**

Antidiabetic sulfonylureas bind to high affinity receptors (SUR) in the plasma membrane of pancreatic B-cells (1). This causes a closure of the plasma membrane ATP-sensitive $K^+$ ($K_{ATP}$) channel (2) and initiates a chain of events that leads to the exocytotic release of insulin (1). Recently, two members of the inward rectifier $K^+$ channel family were cloned, namely Kir6.1 and Kir6.2 (3–5). Together with SUR, which exists in three isoforms—SUR1 and SUR2A/B, they form a functional $K_{ATP}$ channel. For example, SUR1 has been identified as an element composing, together with the inward-rectifier $K^+$ channel Kir6.2, the functional $K_{ATP}$ channel present in the pancreatic B-cell (6, 4). Similar channels are also present in the plasma membrane of smooth, skeletal and cardiac muscle cells as well as in neurons (1). The $K_{ATP}$ channels are specifically activated by drugs known as potassium channel openers (7).

A small conductance $K^+$ channel, with properties similar to the $K_{ATP}$ channel from the plasma membrane, has been described in the inner membrane of rat liver and beef heart mitochondria (mito$K_{ATP}$ channel) (8, 9). The mito$K_{ATP}$ channel is blocked not only by ATP but also by antidiabetic sulfonylureas (for review see (10, 11)) and is activated by some potassium channel openers (12, 13). Recently, using immunofluorescence and immunogold stainings, it has been shown that the inward rectifier $K^+$ channel (Kir6.1) is present in mitochondria (14). This suggests that Kir6.1 may contribute to mito$K_{ATP}$ channel activity.

According to the present knowledge, the mito$K_{ATP}$ channel may have a dual physiological function (for review see (15)). Firstly, it can maintain potassium homeostasis within the mitochondria and thus control mitochondrial volume changes (16). Secondly, potassium uptake upon mitochondrial energization may partly compensate the electric charge transfer produced by proton pumping and thus enable the formation of a pH gradient along with the transmembrane electric potential (17). It was also observed that the mito$K_{ATP}$ channel may be involved in cardioprotection (18, 19).

Recently, the identification and characterization of mitochondrial sulfonylurea receptor (mitoSUR) has been performed with [$^3$H]glibenclamide (20). Glibenclamide binding sites are present both in rat liver and beef heart inner mitochondrial membrane (16, 20). Probably, mitoSUR forms a constituent of the mito$K_{ATP}$ channel (20).

The purpose of the present study was to detect and to characterize thiol (SH) groups in mitoSUR. We describe here the effects of various thiol modifying reagents and substances affecting the redox state of SH groups on [$^3$H]glibenclamide binding to the inner membrane of beef heart mitochondria.

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## MATERIALS AND METHODS

*Preparation of mitochondrial membranes.* Beef heart mitochondria and submitochondrial particles (SMP), a preparation enriched in the inner mitochondrial membrane, were prepared as described previously (16).

*Binding of [$^3$H]glibenclamide to mitochondrial membranes.* The binding of [$^3$H]glibenclamide to SMP membranes was performed as described previously (20). For the equilibrium binding assay, SMP (300–500 $\mu$g protein/ml) were incubated for 60 min. at 4°C in 50 mM Hepes-NaOH (pH 7.4), with the required concentrations of [$^3$H]glibenclamide (usually 2–3 nM, or as otherwise indicated). Incubation was terminated by rapid filtration through Whatman GF/C filters under reduced pressure, followed by washing with 30 ml of 100 mM NaCl, 20 mM Tris-HCl (pH 7.4), at 4°C. Prior to use, the filters were immersed and further incubated for at least 30 min in a solution containing 0.5% polyethylenimine-Hepes (pH 7.4). After use, the filters were incubated for 24 h in 5 ml of scintillation cocktail Formula 989 (Du Pont NEN, Germany) and counted for their associated radioactivity. Non-specific binding was measured in the presence of 30 $\mu$M non-radioactive glibenclamide. To determine apparent $K_D$ values, competition curves were generated using unlabeled glibenclamide over the concentration range from 3 nM to 3 $\mu$M. These data were subsequently converted to Scatchard plots (21, 22).

*Marker enzyme measurements.* The activity of cytochrome c oxidase, a mitochondrial inner membrane marker enzyme, was measured as described by Storrie and Madden (23). The cytochrome c oxidase activity measurements were performed both in the presence and absence of detergent W-1 (Sigma, Germany) and thus allowed estimation of inside-out vesicle populations in the obtained preparations of SMP.

*Materials.* Glibenclamide was purchased from Research Biochemicals Inc. (Natick, MA). [$^3$H]Glibenclamide, with a specific activity of 48.5 Ci/mmol, was from DuPont NEN (Dreich, Germany). Protein concentration was determined with the use of a Bio-Rad protein-assay-kit. All other chemicals were of the highest purity commercially available.

## RESULTS AND DISCUSSION

Inhibition of the mito$K_{ATP}$ by sulfonylureas (8, 10) suggested the presence in the inner mitochodrial membrane of a protein responsible for specific glibenclamide binding–mitochondrial sulfonylurea receptor (mitoSUR). In fact, a single class of low affinity binding sites for glibenclamide in the inner mitochondrial membrane has been found, with a $K_D$ of 360 nM (for beef heart mitochondria) (20) and $K_D$ of 4 $\mu$M (for rat liver mitochondria) (16). Glibenclamide binding is affected by other sulfonylureas but not by potassium channel openers (20). In both the rat liver and beef heart mitochondria adenine nucleotides and its functional analogs (triazine dyes) were found to produce large inhibition of [$^3$H]glibenclamide binding (20). Photoaffinity labeling of beef heart SMP with [$^{125}$I]glibenclamide revealed a single specifically labeled polypeptide band of 28 kDa that was postulated to represent mitoSUR (20).

The SH/-S-S- equilibrium is known to be a well recognized factor influencing the activity of membrane bound enzymes such as glucose-6-phosphatase (24), cytochrome P450 (25), and aldehyde dehydrogenase (25). Also, some of the mitochondrial membrane proteins, such as nicotinamide nucleotide transhydrogenase (26) or permeability transition pore (27), were shown to be regulated by the status of SH groups. It is known that SH reagents can activate $K^+$ transport in intact mitochondria (28, 29). It was also shown that modification of mitochondrial SH groups can activate ATP and glibenclamide sensitive potassium transport (mito$K_{ATP}$ channel) (30, 31).

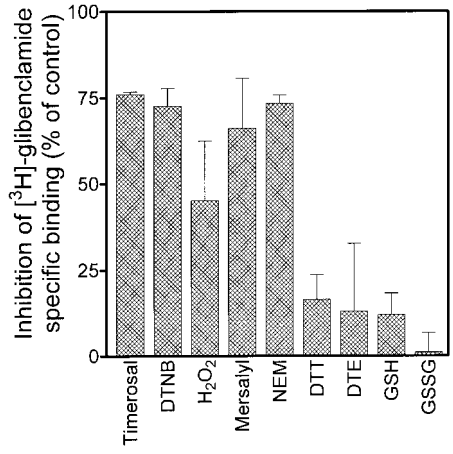


**FIG. 1.** Effect of various SH reagents on [$^3$H]glibenclamide specific binding to the mitochondrial membranes. [$^3$H]glibenclamide specific binding was measured in the presence of 500 $\mu$M timerosal, 500 $\mu$M DTNB, 1% $H_2O_2$, 100 $\mu$M mersalyl, 2 mM NEM, 10 mM DTT, 10 mM DTE, 100 $\mu$M GSH, and 100 $\mu$M GSSG. Measurements were performed in duplicate as described under Materials and Methods. The [$^3$H]glibenclamide concentration was 3 nM.

Figure 1 shows the effects of various thiol reagents on [$^3$H]glibenclamide binding to mitochondrial inner membrane. Thiol oxidizing agents such as timerosal and 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), produced large inhibition (70% to 80%) of specific [$^3$H]-glibenclamide binding to beef heart mitochondria (Fig. 1). A similar effect was observed with thiol modifying agents: NEM and mersalyl (Fig. 1). It has also been found that the binding of [$^3$H]glibenclamide was unaffected by agents such as reduced glutathione$_{(GSH)}$, oxidized glutathione$_{(GSSG)}$, dithiothreitol$_{(DTT)}$ and 2,3-dihydroxy-1,4-dithiolbutane$_{(DTE)}$ (Fig. 1). Glibenclamide binding was affected by hydrogen peroxide and this points to the possibility that at least two SH groups may be important for glibenclamide binding. Figure 2 shows the concentration-dependent effect of thiol reagents on [$^3$H]glibenclamide binding to SMP. The $IC_{50}$ for the described effect is 0.15 ± 0.03 $\mu$M for timerosal, 6.3 ± 3.2 $\mu$M for DTNB, 1.2 ± 0.2 $\mu$M for mersalyl and 0.14 ± 0.05 mM for NEM.

It has been previously reported (20) that the specific binding of [$^3$H]glibenclamide is substantially lowered in the presence of increased concentrations of the unlabeled compound (Fig. 3). Under these conditions, specific binding is estimated to represent 59 ± 7% of the

total binding, the non-specific binding being defined in the presence of 30 $\mu$M glibenclamide. In the presence of 100 $\mu$M mersalyl and 2 mM NEM, the specific binding of [$^3$H]glibenclamide is markedly lower (Fig. 3). Both mersalyl and NEM lower $B_{MAX}$ for glibenclamide from 48 $\pm$ 7 pmol/mg of protein to 12 $\pm$ 1 pmol/mg protein. Concomitantly, $K_D$ values for glibenclamide increased from 0.351 $\pm$ 0.040 $\mu$M, in the absence of thiol modifying agents, to 1.60 $\pm$ 0.8 $\mu$M in the presence of 1 mM NEM and to 2.23 $\pm$ 0.57 $\mu$M in presence of 100 $\mu$M mersalyl.

It is known that the preparation of SMP consists mainly of inside out vesicles. In fact measurements on cytochrome c oxidase activity in intact SMP and in those treated with detergent W-1 has shown that the preparation of SMP applied in our experiments consist of about 75% inside out vesicles and about 25% of non-reversed membranes. It means that the membrane leaflet facing a matrix in intact mitochondria is, to a large extent, accessible to water soluble molecules in the preparation of SMP. Thus the effects observed with membrane permeable (NEM) and non-permeable (mersalyl) sulfhydryl reagents lead to the conclusion that the functionally important SH groups of the mitoSUR in a native system face the mitochondrial matrix.

It has been shown that the mitoSUR may also be considered as an ATP/ADP binding protein (20). Hence, we assessed whether the addition of ATP has a protective effect on SH group modifications. Both in binding studies and in the labeling experiments with [$^{14}$C]N-ethylmaleimide followed by SDS-PAGE electrophoresis and autoradiography, it was not possible to observe a protective effect of 2 mM ATP or 2 mM ADP against the effects of SH reagents on the mitoSUR (data not shown). The properties of the mitoSUR described in this report could be important not only as a potential method of purification of the mitoSUR but also as a reason to raise the question on the possible *in vivo* regulation of the mitoSUR by the overall oxidation state of SH groups. This modulation of mitochondrial protein activities by thiol oxidation is of particular interest in ischemic heart muscle. Ischemia reduces the redox state of the cell and modulates the level of thiol groups of various proteins (32). Thus, it would be interesting to establish whether the mitoSUR or the mito$K_{ATP}$ channel (33) SH groups could be at least partially responsible for some mitochondrial dysfunction during oxidative stress. It is a matter of further investigations to establish the functional role of the mitoSUR SH groups in $K^+$ transport into mitochondria.

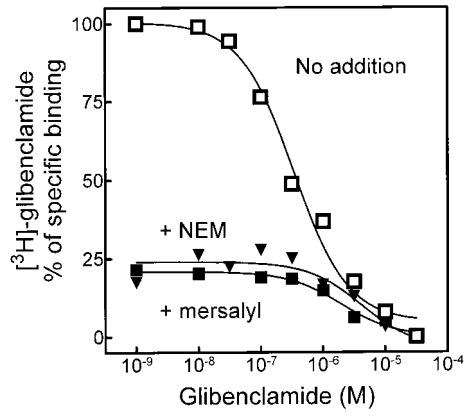


**FIG. 3.** Effect of 100 $\mu$M mersalyl and 1 mM N-ethylmaleimide on specific [$^3$H]glibenclamide binding to beef heart SMP. The [$^3$H]-glibenclamide concentration was 3 nM and the assay conditions were as described under Materials and Methods. The figure shows representative results for 4 different experiments.

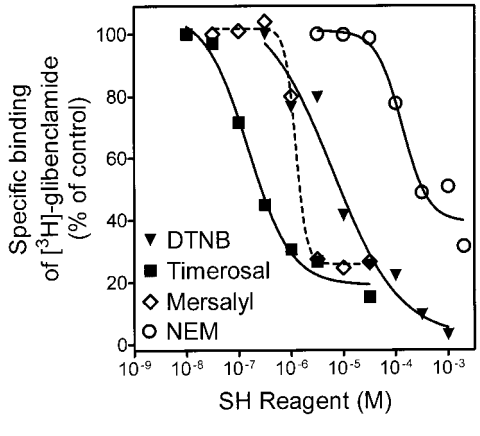


**FIG. 2.** Concentration-dependent effect of SH reagents on specific [$^3$H]glibenclamide binding to mitochondrial inner-membrane. Sample non-treated with SH reagent was used as a reference: 100% of specific binding. [$^3$H]glibenclamide concentration was 3 nM and the assay conditions were as described under Materials and Methods.

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