Inhibition of Succinic Dehydrogenase and F_0F_1-ATP Synthase by 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic Acid (DIDS)

Celene F. Bernardes[a], José R. Meyer-Fernandes[b], Orlando B. Martins[b] and Anibal E. Vercesi[a]

[a] Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, 13084–100, CP 6111, Campinas, SP, Brazil
[b] Departamento de Bioquímica Médica, Universidade Federal do Rio de Janeiro, 21941–590, Rio de Janeiro, RJ, Brazil

Z. Naturforsch. **52c,** 799–806 (1997); received March 18/August 12, 1997

Succinic Dehydrogenase, F_0F_1-ATPase, Thiol Reagent, Oxidative Phosphorylation

This study shows that incubation of rat liver mitochondria in the presence of the thiol/amino reagent 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) is followed by inhibition of both succinate supported respiration and oxidative phosphorylation. Half-maximal inhibition of succinic dehydrogenase activity and succinate oxidation by mitochondria was attained at 55.3 and 60.8 μM DIDS, respectively. DIDS did inhibit the net ATP synthesis and ATP ⇔ [^{32}P]Pi exchange reaction catalyzed by submitochondrial particles in a dose-dependent manner (K_i = 31.7 μM and K_i = 32.7 μM), respectively. The hydrolytic activities of uncoupled heart submitochondrial particles and purified F_1-ATPase were also inhibited 50% by 31.9 and 20.9 μM DIDS, respectively.

Introduction

The disulfonic stilbenes have been described as potent and specific inhibitors of anion transport in red blood cells and several other cellular systems (Cabantchick *et al.*, 1978). Due to their sensitivity to 4,4'-diisothiocyanatostilbene-2,2'disulfonic acid (DIDS) (Lin, 1981; Bennett and Spanswick, 1983; Churchill and Sze, 1983; Xiao-Song *et al.*, 1983; Stone *et al.*, 1984), proton-translocating ATPases present in membrane vesicles from oat or corn roots and brain clathrin-coated vesicles have been characterized as being dependent on chloride as a counterbalancing anion.

More recently DIDS has also been used to modify the chemical structure of several enzymes as an approach to study structure-function relationships (Pedemonte and Kaplan, 1988, 1990; Guilherme *et al.,* 1991). The inhibition of (Na^+ + K^+)ATPase (Pedemonte and Kaplan, 1988, 1990) and (Ca^{2+}+ Mg^{2+})ATPase from the plasma membrane (Guilherme *et al.,* 1991) by this compound seems to involve an interaction of DIDS with amino and/or sulphydryl groups essential for enzyme activity. In this regard, recent work from this laboratory indicates that DIDS interacts with mitochondrial membrane protein thiols creating nonspecific membrane pores by a mechanism stimulated by Ca^{2+} ions (Bernardes *et al.*, 1994). These results indicate that in addition to the use of DIDS as a tool in studies of anion transport and structure-activity relationships in purified systems, this compound would also be important in studies concerning the mechanisms of toxicity of thiols and disulphides in more complex cellular systems. In view of the widespread exposure of man and domestic animals to such compounds, a clear understanding of their toxicity is highly relevant (Munday, 1989).

In the present work we have studied the effect of DIDS on oxidative phosphorylation and mitochondrial respiration. The results obtained with intact rat liver mitochondria led us to investigate the effect of DIDS on the enzymatic activities of succinic dehydrogenase, F_0F_1-ATPase (synthesis and

Abbreviations: CAT, carboxyatractyloside; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HEPES, N-2-hydroxylpiperazine-N'-2-ethanesulfonic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazoly)]benzene); RLM, rat liver mitochondria; SMP, submitochondrial particles; Tris, tris (hydroxymethyl)aminomethane.

Reprint requests to Dr. José R. Meyer-Fernandes.
Fax: +5521 2708647.

0939–5075/97/1100–0799 $ 06.00

hydrolysis reactions) in coupled and uncoupled heart submitochondrial particles (SMP) and purified heart F_1-ATPase.

Material and Methods

Isolation of rat liver mitochondria

Mitochondria were isolated by conventional differential centrifugation from the livers of adult Wistar strain rats fasted overnight. The homogenate was prepared in 250 mM sucrose, 1 mM EGTA and 5.0 mM HEPES buffer, pH 7.2. The mitochondrial suspension was washed twice in the same medium containing 0.1 mM EGTA and the final pellet was diluted in 250 mM sucrose to a protein concentration of 80–100 mg/ml.

Preparation of submitochondrial particles and soluble F_1-ATPase

Bovine heart mitochondria were prepared according to Low and Vallin (1963), and were stored at -70 °C. Mg-ATP submitochondrial particles were prepared as described by Lee and Ernster (1967) and stored under liquid nitrogen until use. F_1-ATPase was prepared from these mitochondria as described by Tuena de Gómes-Puyou and Gómes-Puyou (1977). The enzyme preparation was stored at 4 °C in 2 mM ATP, 2 mM EDTA, and $(NH_4)SO_4$ at 50% saturation. Before use the suspension was centrifuged at 12000 x *g* for 5 min at 4 °C and the pellet dissolved in 10 mM Tris-HCl, pH 8.0, and desalted by centrifuge colunn procedure using Sephadex G-50 previously equilibrated with the same buffer (Kasahara and Penefsky, 1978). Protein concentrations were determined using the Folin phenol reagent (Lowry *et al.*, 1951), and bovine serum albumin as standard.

Oxygen uptake measurements

Oxygen consumption was measured using a Clark-type electrode (Yellow Springs Instruments Co.) in a 1 ml glass chamber equipped with magnetic stirring. The results shown are representative of a series of three experiments.

Assay of succinic dehydrogenase

Succinic dehydrogenase activity was measured spectrophotometrically in the presence of Rat liver mitochondria (RLM 1 mg/ml), 0.05% Triton X-100, 1 μM antimycin A, 5 mM succinate, 100 μM 2,6-dichlorophenol indophenol and 2 mM phenazine methosulfate, in a final volume of 1.0 ml (30 °C) at 600 nm (Veeger *et al.*, 1969). The values shown represent the average ± SE of three different experiments.

Assay of net ATP synthesis

Net synthesis of ATP from [^{32}P]Pi and ADP was measured by coupling the production of ATP to the phosphorylation of glucose by hexokinase (Vercesi *et al.*,1990). The glucose 6-[^{32}P]phosphate so produced was measured in the aqueous phase after extraction of the excess [^{32}P]Pi as described by Vieyra *et al.* (1991). The basic medium for net synthesis contained 65 mM Tris-maleate buffer, 4 mM potassium [^{32}P]phosphate (spec. act. about 10^5 Bq/nmol Pi), 1 mM ADP, 0.2 mM EGTA, 10 mM AMP, 100 μM adenosine pentaphosphate, 5 μM CAT, 26 units hexokinase, 20 mM glucose, 10 mM α-ketoglutarate and 50 μg/ml SMP protein, in a final volume of 1.0 ml at pH 7.5 and 37° C. The reaction was initiated by the addition of 10 mM $MgCl_2$ after 5 min preincubation of the particles in this reaction medium, and were stopped 5 min later by the addition of a solution of ammonium molybdate in 5 N sulfuric acid. The excess [^{32}P]Pi was removed as the phosphomolybdate complex using six consecutive extractions with acetone plus butyl acetate, leaving the glucose 6-[^{32}P]phosphate in the aqueous phase (Vieyra *et al.*, 1991). 400 μl of these molybdate extracts were added to 9 ml of scintillation liquid (2 g PPO, 1 g POPOP in 1 l toluene) and counted in a liquid scintillation counter. The values shown represent averages ± SE of three different experiments.

Assay of ATP ⇔ [^{32}P]Pi exchange activity

The exchange reaction was assayed by measuring the formation of [γ^{32}P]ATP from ADP and [^{32}P]Pi. The basic medium for the ATP ⇔ [^{32}P]Pi exchange contained 65 mM Tris-maleate buffer, 4 mM potassium [^{32}P]phosphate (spec. act. about 10^5 Bq/nmol Pi), 1 mM ADP, 1 mM ATP, 0.2 mM EGTA, 5 μM CAT and 50 μg/ml SMP protein, in a final volume of 0.5 ml at pH 7.5 and 37° C. The reactions were iniated by the addition of 10 mM $MgCl_2$, after 5 min preincubation of the particles

in the reaction medium, and stopped 5 min later by the addition of ammonium molybdate in 5 N sulfuric acid. The excess [^{32}P]Pi was removed as the phosphomolybdate complex using six consecutive extractions with acetone plus butyl acetate, leaving the [γ^{32}P]ATP in the aqueous phase (Vieyra *et al.*, 1991). 400 µl of these molybdate extracts were counted in a liquid scintillation counter. The values shown represent averages ± SE of three different experiments.

Assay of ATPase activity

ATPase activity was assayed by measuring the hydrolysis of [γ^{32}P]ATP (specific activity about 10^4 Bq/nmol ATP). The reaction mixture contained 65 mM Tris-maleate buffer, 1 µM FCCP, 5 mM ATP, 20 mM KCl, 0.2 mM EGTA, 1 µg/ml F_1-ATPase or 10 µg/ml SMP protein, in a final volume of 0.5 ml at pH 7.5 and 37 °C. The reactions were initiated by the addition of 10 mM $MgCl_2$, after 5 min preincubation of the particles or the F_1-ATPase in the reaction medium, and were stopped 5 min later by the addition of 1 ml of a cold mixture containing 0.2 g charcoal in 1 N HCl (Vercesi *et al.*, 1990). The tubes were then centrifuged at 1500 x *g* for 20 min at 4 °C, after which 400 µl of the supernatant was added to 9 ml of scintillation liquid and counted in a liquid scintillation counter. Under these conditions the reactions follow a linear time-course during the period of assay. The values shown represent averages ± SE of three different experiments.

Reagents and solutions

[γ^{32}P]Pi was obtained from the Brazilian Institute of Atomic Energy and purified by extraction as the phosphomolybdate complex with a mixture of 2-butanol/benzene, followed by reextraction to the aqueous phase with ammonium hydroxide and precipitation as the $MgNH_4PO_4$ salt (De Meis, 1984). The isotope was stored at room temperature in 10 mM HCl. It was mixed with nonradioactive potassium phosphate and neutralized with Tris base just before use. [γ^{32}P]ATP was prepared according to Glynn and Chappell (1964) and stored at -70 °C. Nucleotides, DIDS, CAT, EDTA, EGTA, FCCP, and hexokinase were from Sigma Chemical Co., St Louis, MO. Other reagents were of analytical grade.

Results

Inhibition by DIDS of succinic dehydrogenase in isolated rat liver mitochondria

Fig. 1 shows the rates of FCCP-induced state-3-respiration supported by α-ketoglutarate (A), succinate (B) or N,N,N,N-tetramethyl-*p*-phenylenediamine (TMPD) (C) oxidation, in the presence or absence of DIDS. Important inhibition of respiration by DIDS was observed only when the substrate was succinate. This inhibition was dose-dependent; 50% inhibition was attained at a DIDS concentration of 60.8 µM. The same degree of inhibition was observed in mitochondria permeabilized with triton X-100 (Fig. 2). This indicates that succinate transport was not the cause of this inhibition. This interpretation was further supported by Table I showing that the activity of the succinic dehydrogenase was also inhibited by the same concentrations of DIDS (K_i = 55.3 µM).

Inhibition by DIDS of ADP-induced state-3-respiration in intact rat liver mitochondria

Fig. 3 shows that in the absence of DIDS, the addition of ADP caused the expected state-4, state-3 respiratory transitions in intact mitochondria respiring on α-ketoglutarate. This was completely inhibited by 75 µM DIDS.

Inhibition by DIDS on net ATP synthesis and ATP ⇔ [^{32}P]Pi exchange reactions by coupled heart submitochondrial particles

In order to verify whether inhibition of oxidative phosphorylation by DIDS could be mediated by interactions of the compound with the F_0F_1-ATP synthase, experiments were performed measuring the net ATP synthesis catalyzed by energized submitochondrial particles. As shown in Table I, this compound did inhibit the net ATP synthesis in a dose dependent manner (K_i = 31.7 µM). Net ATP synthesis and ATP ⇔ [^{32}P]Pi exchange have different responses towards the same ligands, and this has been ascribed to differences in the mechanism by which the particles are energized: by electron transport or by ATP hydrolysis (Gomez-Puyou *et al.*, 1984). For this reason, the effect of DIDS on ATP ⇔ [^{32}P]Pi exchange was also studied. Table I shows that as the net synthesis, DIDS did also inhibit the ATP ⇔

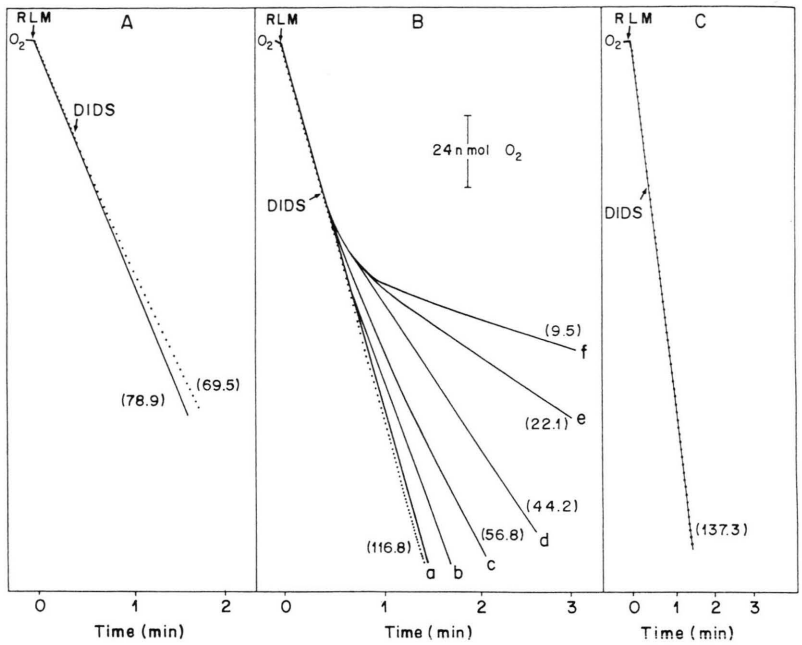

Fig. 1. Oxygen consumption by uncoupled rat liver mitochondria in the presence of DIDS. Rat liver mitochondria (RLM,1 mg/ml) were added to a reaction medium (1.3 ml, 30 °C) containing 125 mM sucrose, 65 mM KCl, 10 mM Tris/HCl buffer pH 7.2, 10 μM Ca^{2+} and 1 μM FCCP plus: 5 mM α-ketoglutarate (panel A); 5 mM succinate and 5 μM rotenone (panel B) or 0.5 mM TMPD, 1 mM ascorbate and 1 μg/ml antimycin A (panel C). DIDS (100 μM, panel A and C) or (10, 25, 50, 75, 100 and 200 μM, panel B, lines a-f, respectively) was added where indicated. The dotted lines represent experiments in the absence of DIDS. The numbers in parentheses indicate the rate of oxygen uptake in ng-atoms O/min/mg of protein.

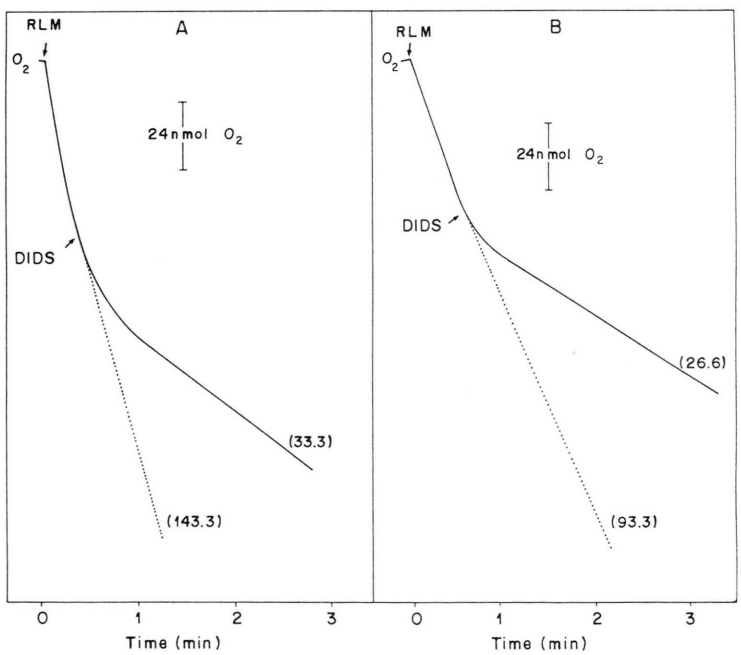

Fig. 2. Inhibition by DIDS of succinate supported oxygen consumption by rat liver mitochondria treated with Triton X-100. The experimental conditions were as in FIG. 1B, except that 0.01% Triton X-100 (panel B) or 1 μM FCCP (panel A) were present. DIDS (100 μM) was added where indicated.

Table I. Inhibition by DIDS of succinic dehydrogenase and F_1ATPase activities.

DIDS [μM]	Succinic dehydrogenase	ATP Synthesis	ATP ⇔ [^{32}P]Pi	ATP Hydrolysis (SMP)	ATP Hydrolysis (F_1ATPase)
0	100.0 ± 15.1	100.0 ± 7.8	100.0 ± 6.9	100.0 ± 12.1	100.0 ± 9.2
10	86.1 ± 7.8	81.3 ± 8.2	81.0 ± 7.8	82.0 ± 4.9	74.0 ± 7.8
50	57.2 ± 3.9	39.0 ± 2.9	42.0 ± 3.5	41.0 ± 3.1	27.0 ± 2.9
100	29.4 ± 3.6	20.1 ± 2.1	21.4 ± 1.6	19.5 ± 2.1	19.4 ± 1.9
200	3.9 ± 0.1	9.9 ± 0.6	9.6 ± 0.9	10.0 ± 0.7	9.8 ± 0.1

The enzyme activities were measured as indicated under material and methods. 100% activity corresponds to 76.1 ± 11.5 nmol Succinate oxidized . mg^{-1} . min^{-1} for succinic dehydrogenase activity, 66.9 ± 5.2 nmol ATP . mg^{-1} . min^{-1} for ATP net synthesis, 59.6 ± 4.1 nmol ATP . mg^{-1} . min^{-1} for ATP ⇔ [^{32}P]Pi exchange reaction, 1.6 ± 0.2 μmol Pi . mg^{-1} . min^{-1} for ATP hydrolysis catalyzed by SMP and 47.4 ± 4.4 μmol Pi . mg^{-1} . min^{-1} for ATP hydrolysis catalyzed by.purified F_1ATPase. The standard errors were calculated from the absolute activity values of three different experiments and converted to percentage of the control value.

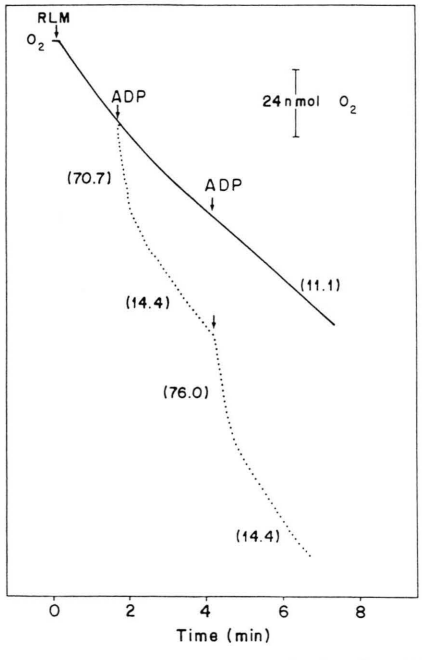

Fig. 3. Inhibition of ADP-stimulated mitochondrial oxygen consumption by DIDS. Rat liver mitochondria (RLM, 1 mg/ml) were added to a medium (1.3 ml, 30 °C) containing 125 mM sucrose, 65 mM KCl, 10 mM Tris/HCl buffer pH 7.2, 5 mM α-ketoglutarate, 2 mM potassium phosphate, 10 μM Ca^{2+} and 75 μM DIDS. ADP (200 nmol) was added where indicated. The dotted line represents an experiment without DIDS. The numbers in parentheses indicate the rate of oxygen uptake in ng-atoms O/min/mg protein.

[^{32}P]Pi exchange reaction in a same dose dependent manner (Ki = 32.7 μM).

Inhibition by DIDS of ATP hydrolysis by uncoupled heart submitochondrial particles or by purified F_1-ATPase

DIDS inhibition of ATP synthesis by ATP ⇔ [^{32}P]Pi exchange reaction, where the particles are energized by ATP hydrolysis (Gomez-Puyou *et al.*, 1984), suggests that the inhibition of oxidative phosphorylation by DIDS could be mediated by interactions of the compound with the F_0F_1-ATP synthase. Experiments were performed measuring the hydrolytic activities of both heart submitochondrial particles and purified F_1-ATPase. Table I shows that ATP hydrolysis catalyzed by submitochondrial particles was inhibited by increasing concentrations of DIDS; half-maximal inhibition occurred at about 31.9 μM DIDS. In order to determine whether the inhibitory effect of DIDS on ATP synthesis and Hydrolysis was caused by a direct interaction with the F_1-ATPase, the rate of ATP hydrolysis was measured with the purified enzyme at different DIDS concentrations. As is the case for ATP hydrolysis catalyzed by submitochondrial particles, the ATPase activity of the soluble enzyme was inhibited by DIDS (Table I). However, the hydrolytic activity of soluble enzyme appears to be more sensitive (K_i = 20.9) than the hydrolytic activity catalyzed by submitochondrial particles (K_i = 31.9). These date could be indicat-

ing that the procedure of the purification, may alter the ATPase preparation in a manner that increases its interactions with DIDS. It has been shown that inhibition by DIDS of solubilized (Ca^{2+}+Mg^{2+})ATPase from plasma membrane is more sensitive than the pumping activity catalyzed by membrane-bound enzyme (Guilherme *et al.*, 1991).

Discussion

The negatively charged amino/sulphydryl-reactive reagent DIDS has been widely employed as a tool to study anion transport (Cabantchik, 1978; Zaki *et al.*, 1975; Leptke *et al.*, 1976; Kasai and Taguchi, 1981) and to identify possible coupling between the flux of anions and the transport of cations such as H^+ (Lin, 1981; Bennett and Spanswick, 1983; Churchill and Sze, 1983; Xiao-Song *et al.*, 1983; Stone *et al.*, 1984) or Ca^{2+} (Campbell and McLennan, 1981; Waisman *et al.*, 1981, 1982; Romero and Ortiz, 1988). In spite of this, other data suggest that this compound may also interact with other enzymes not involved in the translocation of anions (Pedemonte and Kaplan, 1988, 1990; Guilherme *et al.*, 1991). Indeed, previous data from this laboratory provided evidence that inner mitochondrial membrane permeabilization by Ca^{2+} ions in the presence of DIDS is mediated by DIDS reaction with membrane protein thiols (Bernardes *et al.*, 1994). We now show that two mitochondrial enzymes (succinic dehydrogenase and F_1-ATPase), not involved in anion transport, are sensitive to DIDS. The first one has sulphydryl groups that may react with DIDS as they do with *N*-ethylmaleimide (Kenney, 1975; Birch-Machin *et al.*, 1992) and with peroxynitrite (Denicola *et al.*, 1993). Regarding the inhibition of oxidative phosphorylation, the effect of DIDS on ATP net synthesis and ATP $\Leftrightarrow$ [^{32}P]Pi exchange reactions by coupled heart submitochondrial particles but also on ATP hydrolysis by either SMP particles or purified F_1ATPase (Table I) suggests that the compound may also interact with thiols or amino groups (Guilherme *et al.*, 1991) present in the F_1 moiety of the enzyme. Therefore, the inhibition of ATP synthesis in intact mitochondria (Fig. 3) seems to be mediated by direct inhibition of ADP phosphorylation at the level of the F_1-ATPase. Indeed , literature data indicate that the ATP-synthase have two or more sets of dithiol-disulfides whose oxidation-reduction states are essential to energy transfer (Yagi and Hatefi, 1984, Hatefi, 1985; Grüber and Capaldi, 1996). It was recently shown that the disulfide bond formation between γ and ε subunits induced by $CuCl_2$ in *Escherichia coli* F_1 -ATPase totally inhibited the ATPase activity (Haughton and Capaldi, 1995; Aggeler and Capaldi, 1996; Grüber and Capaldi, 1996). The inhibition of ATP hydrolysis catalyzed by purified F_1-ATPase observed in this work may result from the covalent reaction of DIDS (Bernardes *et al.*, 1994) with thiols present in the α, γ and ε subunits of F_1-ATPase (Zanotti *et al.*, 1992; Haughton and Capaldi, 1995; Aggeler and Capaldi, 1996; Grüber and Capaldi, 1996).

In addition, it can be concluded from the data presented here that: (1) DIDS can be used as a tool to study structure-function relationships in succinic dehydrogenase and F_0F_1-ATP synthase and; (2) the interpretation that some anions are cotransported by H^+-ATPases on the basis of ATPase sensitivity to DIDS should be revised.

Acknowledgements

We thank Dr. Sergio T. Ferreira and Alicia J. Kowaltowski for critical reading of the manuscript and Mr. Luis H. G. Ribeiro for excellent technical assistance. This work was partially supported by grants from the Brazilian Agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Programa de Apoio ao Desenvolvimento Científico e Tecnológico (PADCT), Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), Financiadora de Estudos e Projetos (FINEP) and FAEP-UNICAMP.

Aggeler R. and Capaldi. R. A. (1996), Nucleotide-dependent movement of the ε subunit between α and β subunits in the *Escherichia coli* F_1F_0-type ATPase. Biol. Chem. **271**, 13888–13891.

Bennett A. B. and Spanswick R. M. (1983), Optical measurements of ΔpH and $\Delta\Psi$ in corn root membrane vesicles: Kinetic analysis of Cl^- effects on a proton-translocating ATPase. J. Membr. Biol. **71**, 95–107.

Bernardes C. F., Meyer-Fernandes J. R., Basseres D. S., Castilho R. F. and Vercesi A. E. (1994), Ca^{2+}-dependent permeabilization of inner mitochondrial membrane by 4,4'diisothiocyanatostilbene-2,2'disulfonic acid (DIDS). Biochim. Biophys. Acta **1188**, 93–100.

Birch-Machin M. A., Farnsworth L., Ackrell B. A. C., Cochran B., Jackson S., Bindoff L. A., Aitken A., Diamond A. G. and Turnbull D. M. (1992), The sequence of the flavoprotein subunit of bovine heart succinate dehydrogenase. J. Biol. Chem. **267**, 11553–11558.

Cabantchik Z. I., Knauf P. A. and Rothstein A. (1978), The anion transport system of the red blood cell. The role of membrane protein evaluated by the use of probes. Biochim. Biophys. Acta **515**, 239–302.

Campbell K. P. and McLennan D. H. (1980), DIDS inhibition of sarcoplasmic reticulum anion efflux and calcium transport. Ann. N. Y. Acad. Sci. **358**, 328–331.

Churchill K. A. and Sze H. (1983), Anion-sensitive H^+-pumping ATPase in membrane vesicles from oat roots. Plant Physiol. **71**, 610–617.

de Meis L. (1984), Pyrophosphate of high and low energy. J.Biol.Chem. **259**, 6090–6097.

Denicola A., Rubbo H., Rodrigues D. and Radi R. (1993), Peroxynitrite-mediated cytoxicity to *Trypanosoma cruzi*. Arch. Biochem. Biophys. **304**, 279–286.

Glynn I. M. and Chappell J. B. (1964), A simple method for the preparation of ^{32}P-labelled Adenosine triphosphate of high specific activity. Biochem. J. **90**, 147–149.

Gomez-Puyou M. T., Ayala G., Darszon A. and Gomez-Puyou A. (1984), Oxidative Phosphorylation and the Pi – ATP exchange reaction of submitochondrial particles under the influence of organic solvents. J. Biol. Chem. **259**, 9472–9478.

Grüber G. and Capaldi R. A. (1996), The trapping of different conformations of the *Escherichia coli* F_1 ATPase by disulfide bond formation. J. Biol. Chem. **271**, 32623–32628.

Guilherme A., Meyer-Fernandes J. R. and Vieyra A. (1991), Reversible inhibition by 4–4'-diisothiocyanatostilbene-2–2-disulfonic acid of the plasma membrane (Ca^{2+}+Mg^{2+}) ATPase from kidney proximal tubules. Biochemistry **30**, 5700–5706.

Hatefi Y. (1985), The mitochondrial electron transport and oxidative phosphorylation system. Annu. Rev. Biochem. **54**, 1015–1069.

Haughton M. A. and Capaldi R. A. (1995), Asymmetry of *Escherichia coli* F_1-type ATPase as a function of the interaction of α-β subunit pairs with the γ and ε subunits. J. Biol. Chem. **270**, 20568–20574.

Kasahara M. and Penefsky H. S. (1978), High affinity binding of monovalent Pi by beef heart mitochondrial adenosine triphosphate. J. Biol. Chem. **253**, 4180–4187.

Kasai M. and Taguchi T. (1981), Inhibition of anion permeability of sarcoplasmic reticulum vesicles by stilbene derivates and the identification of an inhibitor-binding protein. Biochim. Biophys. Acta **643**, 213–219.

Kenney W. C. (1975), The reaction of *N*-Ethylmaleimide at the active site of Succinate dehydrogenase. J. Biol. Chem. **250**, 3089–3094.

Lee C-P. and Ernster L. (1967), Energy-coupling in non-phosphorylating submitochondrial particles. Methods Enzymol. **10**, 543–548.

Leptke S., Fasold H., Pring M. and Passow H. (1976), A study of the relationship between inhibition of anion exchange and binding to the red cell membrane of 4,4'diisothiocyanatostilbene-2,2'disulfonic acid (DIDS) and its dihydroderivative (H_2DIDS). J. Membr. Biol. **29**, 147–177.

Lin W. (1981), Inhibition of anion transport in corn root protoplasts. Plant Physiol. **68**, 435–438.

Löw H. and Vallin I. (1963), Succinate-linked diphosphopyridine nucleotide reduction in submitochondrial particles. Biochim. Biophys. Acta **69**, 361–374.

Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J. (1951), Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**, 265–275.

Munday R. (1989), Toxicity of thiols and disulphides: involvement of free radical species. Free Rad. Biol. & Med **7**, 659–673.

Pedemonte C. H. and Kaplan J. H. (1988), Inhibition and derivatization of the renal Na,K-ATPase by dihydro-4,4'diisothiocyanatostilbene-2,2'disulfonate. Biochemistry **27**, 7966–7973.

Pedemonte C. H. and Kaplan J. H. (1990), Chemical modification as an aproach to elucidation of sodium pump structure-function relations. Am. J. Physiol. **258**, C1-C23.

Romero P. J. and Ortiz C. (1988), Eletrogenic behavior of the human red cell Ca^{2+} pump revealed by disulfonic stilbenes. J. Membr. Biol. **101**, 237–246.

Stone D. K., Xiao-Song X. and Racher E. (1984), Inhibition of clathrin-coated vesicle acidification by duramycin. J. Biol. Chem. **259**, 2701–2703.

Tuena de Gómes-Puyou M. and Gómes-Puyou A. (1977), A simple method of purification of a soluble oligomycin-insensitive mitochondrial ATPase. Arch. Biochem. Biophys. **182**. 82–86.

Veeger C., Dervartanian D. V. and Zeylemaker W. P. (1969), Succinate dehydrogenase. Methods Enzymol. **13**, 81–83.

Vercesi A. E., Hermes-Lima M., Meyer-Fernandes J. R. and Vieyra A. (1990), Calcium inhibition of the ATP ⇔ [^{32}P] Pi exchange and net ATP synthesis catalyzed by bovine submitochondrial particles. Biochim. Biophys. Acta 1020, 101–106.

Vieyra A., Caruso-Neves C. and Meyer-Fernandes J. R. (1991), ATP ⇔ ^{32}Pi exchange catalyzed by plasma membrane Ca^{2+}-ATPase from kidney proximal tubules. J.Biol. Chem. **266**, 10324–10330.

Waisman D. M., Gimble J. M., Goodman D. B. P. and Rasmussen H. (1981), Studies of the Ca^{2+} transport mechanism of human erytrocyte inside-out plasma membrane vesicles. II. Stimulation of the Ca^{2+} pump by phosphate. J. Biol. Chem. **256**, 415–419.

Waisman D. M., Smallwood J., Lafreniere D. and Rasmussen H. (1982), The role of band III in calcium transport across the human erythrocyte membrane. FEBS Lett. **145**, 337–340.

Xiao-Song X., Stone D. K. and Racher E. (1983), Determinants of clathrin-coated vesicle acidification. J. Biol. Chem. **258**, 14834–14838.

Yagi T. and Hatefi Y. (1984), Thiols in oxidative phosphorylation: Inhibition and energy-potentiated uncloupling by monothiol and dithiol modifiers. Biochemistry **23**, 2449–2455.

Zaki L., Fasold H., Schuhmann D. and Passow H. (1975), Chemical modification of membrane proteins in relation to inhibition of anion exchange in human red blood cells. J. Cell. Physiol. **86**, 471–494.

Zanotti F., Guerrieri F., Capozza G., Fiermonte M., Berden J. and Papa S. (1992), Role of F_0 and F_1 subunits in the gating and coupling fucntion of mitochondrial H^+-synthase. The effect of dithiol reagents. Eur. J. Biochem. **208**, 9–16.