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N-Ethylmaleimide and mercurials modulate inhibition of the mitochondrial inner membrane anion channel by H^+, Mg^{2+} and cationic amphiphiles

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Previously it has been shown that the mitochondrial inner membrane anion channel is reversibly inhibited by matrix Mg^{2+}, matrix H^+ and cationic amphiphiles such as propranolol. Furthermore, the IC_{50} values for both Mg^{2+} and cationic amphiphiles are dependent on matrix pH. It is now shown that pretreatment of mitochondria with *N*-ethylmaleimide, mersalyl and *p*-chloromercuribenzenesulfonate increases the IC_{50} values of these inhibitors. The effect of the mercurials is most evident when cysteine or thioglycolate is added to the assay medium to reverse their previously reported inhibitory effect (Beavis, A.D. (1989) Eur. J. Biochem. 185, 511–519). Although the IC_{50} values for Mg^{2+} and propranolol are shifted they remain pH dependent. Mersalyl is shown to inhibit transport even in *N*-ethylmaleimide-treated mitochondria indicating that *N*-ethylmaleimide does not react at the inhibitory mercurial site. However, the effects of *N*-ethylmaleimide and mersalyl on the IC_{50} for H^+ are not additive which suggests that mercurials and *N*-ethylmaleimide react at the same 'regulatory' site. It is suggested that modification of this latter site exerts an effect on the binding of Mg^{2+}, H^+ and propranolol by inducing a conformational change. It is also suggested that a physiological regulator may exist which has a similar effect in vivo.

Introduction

The inner membrane anion channel (IMAC) is a transport pathway which catalyzes the electrophoretic uniport of a variety of anions across the inner mitochondrial membrane [1,2]. Although the existence of an anion uniport pathway has been recognized for a long time [3–6], it is only in recent years that it has become apparent that a specific protein may be involved.

IMAC is characterized by the following properties: It is reversibly inhibited by matrix protons [1], magnesium [1,7] and cationic amphiphilic drugs such as propranolol [8]. Moreover, the IC_{50} values for Mg^{2+} [7] and propranolol [8] increase as the pH increases in a manner which suggests that matrix protons modulate inhibition by these agents. IMAC is also irreversibly inhibited by reaction with the alkylating agent *N*,*N*′-dicyclohexylcarbodiimide [9,10]. Selwyn's group has recently shown that anion uniport is also inhibited by agaric acid [11], bongkrekic acid [11], palmitoyl-CoA [12,13] and Cibacron blue [11]. Since these are all inhibitors of the adenine nucleotide translocase, they have suggested that the two proteins may be genetically related [11]. This would make IMAC a member of a protein family which Aquila et al. [14] have proposed includes the adenine nucleotide translocator, the phosphate carrier and the uncoupling protein of brown fat mitochondria.

Although not unique to this 'family', one property that these three proteins have in common is that each has sulfhydryl groups which are functionally important [15–26]. Consistent with Selwyn's proposal, I have recently presented evidence that IMAC may also have important groups which react with mersalyl and *p*-CMS [27]. Two binding sites appear to exist: Reaction at site 1 induces a decrease in the pIC_{50} for protons which leads to stimulation of transport at neutral pH. Reaction at site 2 induces complete inhibition of malonate

Abbreviations: EGTA, [ethylene bis (oxyethylenenitrilo)]-tetraacetic acid; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; Tes, *N*-tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid; *p*-CMS, *p*-chloromercuribenzenesulfonate; NEM, *N*-ethylmaleimide; IMAC, inner membrane anion channel.

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uniport and partial inhibition of Cl^- uniport. These two independent effects are distinguished by the finding that inhibition is reversed by cysteine and thioglycolate, whereas the decrease in the pIC_{50} is not. In contrast to mersalyl and *p*-CMS, NEM, an inhibitor of the adenine nucleotide translocator [15–17] and the phosphate carrier [28], does not inhibit anion uniport [1,27]; however, since the extent of inhibition by mercurials appears to depend on the specific reagent used [27], lack of inhibition does not rule out the possibility that NEM may react with one or both sites. In fact, Rial et al. [25] have recently presented evidence that NEM stimulates GDP-insensitive anion uniport in brown adipose tissue mitochondria and, on the basis of previous reports [2,29,30], have suggested that this may reflect activation of IMAC in these mitochondria.

In the present paper, I present evidence which suggests that NEM binds to mersalyl binding site 1 but not to site 2. I also show that reaction of NEM and mercurials at site 1 not only modifies the pH dependence of transport but also modulates inhibition of IMAC by magnesium and propranolol. These results provide evidence that a thiol group may play an important role in the regulation of IMAC. Some of these data have been presented in a preliminary form [31].

Materials and Methods

Assay of anion transport. Anion transport was assayed by following swelling, which accompanies net salt transport, using the light scattering technique as described in detail elsewhere [32,33].

Treatment of mitochondria with NEM. Rat liver mitochondria (50 mg/ml) were treated with the desired amount of NEM in the stock suspension which contained 250 mM sucrose and was supplemented with the K^+ salts of Tes (5 mM), EGTA (0.2 mM) and rotenone (0.5 μg/mg) and adjusted to pH 6.7 (at 25°C). After addition of NEM the suspension was kept on ice for 10 min and then, unless indicated otherwise, cysteine (10 mM) was added to terminate the reaction.

Pretreatment with mersalyl and p-CMS. The normal mitochondrial stock suspension (50 mg protein/ml) was diluted 1:1 in 0.25 M sucrose containing K^+ salts of Tes (12 mM) and EGTA (0.5 mM) adjusted to pH 6.7 (at 25°C) and maintained at 0°C. The desired dose of mercurial was then added and at least 1 min was allowed to elapse after mixing before the mitochondria were transferred to the various assay media.

Assay medium for anion transport. The potassium chloride, malonate and thiocyanate media for light scattering studies contained the K^+ salts of Cl^- (55 mM) or SCN^- (55 mM) or malonate (36.7 mM) and EDTA (0.1 mM), EGTA (0.1 mM) and Tes (5 mM) plus rotenone (2 μg/mg). The pH was adjusted to 7.4 unless indicated otherwise and the temperature was maintained at 25°C.

Results

NEM stimulates uniport of chloride

Fig. 1A shows typical light scattering traces of nonrespiring mitochondria suspended in KCl assay medium. The medium included valinomycin to provide a pathway for K^+ uniport and nigericin to buffer the pH gradient (see Ref. 1). Under these conditions, the rate of net salt transport and associated swelling is limited by the permeability to Cl^-. Thus, the stimulation induced by the addition of A23187, an ionophore which mediates $Mg^{2+}/2H^+$ antiport, reflects activation of IMAC fol-

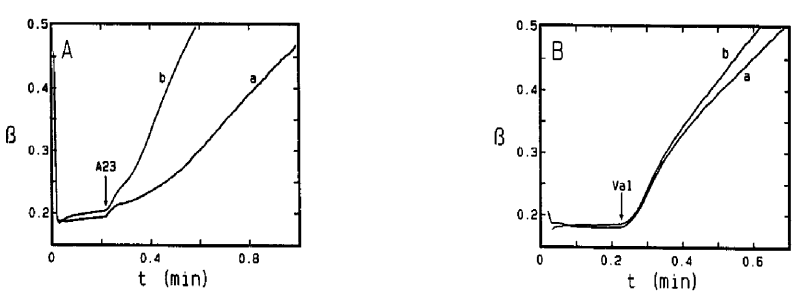

Fig. 1. *N*-Ethylmaleimide stimulates the transport of Cl^-. Light scattering kinetics of mitochondria (0.1 mg/ml) suspended in KCl (Panel A) and KSCN (Panel B) assay media are shown. β is a light scattering parameter, defined

$$\beta \equiv \frac{P}{P_s}(A^{-1} - a)$$

which normalizes reciprocal absorbance for mitochondrial concentration, (P(mg/ml)), where $P_s = 1$ mg/ml and a is a machine constant equal to 0.25 with our apparatus [32,33]. Trace a control mitochondria. Trace b NEM-treated (27 nmol/mg) mitochondria. (A) Valinomycin (0.5 nmol/mg), nigericin (1 nmol/mg) and rotenone (2 μg/mg) were included in the medium. A23187 (A23, 10 nmol/mg) was added at 0.2 min to activate IMAC. The immediate small increase in A reflects net exchange of matrix Mg^{2+} for medium K^+, and after a short lag maximum uniport activity is seen [1]. Rates are compared at equal values of β (equal matrix volumes). (B) Rotenone (2 μg/mg) was included in the medium and valinomycin (Val, 0.5 nmol/mg) was added at 0.2 min. See Materials and Methods for details of pretreatment with NEM and composition of the assay media.

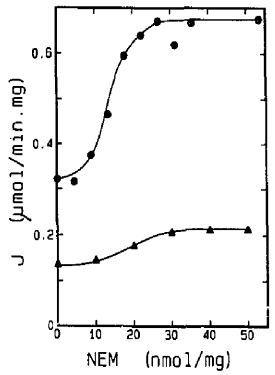

Fig. 2. Dose-response curves for stimulation of anion uniport by NEM. The rates of Cl^- uniport (●), and malonate uniport (▲) are plotted versus the dose of NEM. Rates of transport were determined from light scattering kinetics, similar to those shown in Fig. 1, as described in Refs. 32 and 33. The composition of the assay media and the procedure used for pretreatment of the mitochondria with NEM are described under Materials and Methods.

lowing depletion of matrix divalent cations which inhibit this transport pathway [1]. Trace a shows the result of an experiment using normal mitochondria and trace b shows the result obtained using mitochondria pretreated with NEM. Clearly, NEM stimulates the rate of A23187-induced Cl^- transport.

Since the rate of transport can be affected by the salt gradient as well as by the activity of the carrier [32,33], it was important to determine whether the effect was specific for IMAC-mediated transport. Fig. 1B shows the results of an experiment in which transport of KSCN induced by the addition of valinomycin was followed. Since no A23187 was added almost all the transport observed should be through the lipid bilayer [1]. In this case, the effect of NEM on the initial rate is negligible; however, the later rate is increased by about 10–15%. A similar result was obtained in KCl using nigericin and tributyltin, which catalyzes Cl^-/OH^- exchange, to induce swelling (not shown). The absorbance osmotic curves [32] obtained with normal and NEM-treated mitochondria were also compared to determine whether the solute content of the NEM-treated mitochondria might be higher. Although an increase in solute content should not affect the initial rate, it should slow the decline in the gradient and rate of transport. Consistent with the data in Fig. 1B a small increase of about 8–10% in the slope was detected (data not shown). Since the effect of NEM on IMAC-mediated transport is much greater than that on SCN^- transport and tributyltin-mediated Cl^- transport through the lipid bilayer, we conclude that NEM is acting on IMAC.

The data contained in Fig. 2 show dose-response curves for both Cl^- transport and malonate transport. NEM stimulates the transport of each anion about 2-fold with the maximum stimulation being obtained with about 30 nmol/mg. No further increase was observed when the dose was increased up to 90 nmol/mg. For most subsequent studies doses between 30 and 50 nmol/mg were routinely used.

NEM shifts the pH dependence of anion uniport

In a previous study [27], I showed that the mercurial mersalyl decreases the proton pIC_{50} for anion uniport. Consequently, to determine whether the stimulation of anion uniport by NEM was effected by a similar mechanism or by direct activation of the uniporter, I investigated the pH dependence of Cl^- uniport in NEM-treated mitochondria. In order to determine the $J_{max}([H^+] = 0)$ of transport, $1/J$ was plotted versus

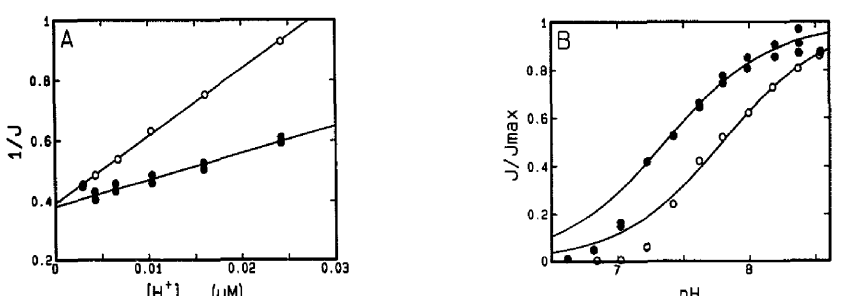

Fig. 3. Effect of NEM on the regulation of Cl^- uniport by protons. The rate of Cl^- uniport (J, μmol Cl^-/min per mg) in mitochondria (0.1 mg/ml) pretreated with or without NEM (50 nmol/mg) was assayed using the light scattering technique in KCl assay media adjusted to the indicated pH values. (A) $1/J$ is plotted versus $[H^+]$ for the pH values above 7.5. From this linear relationship the value of J_{max} ($[H^+] = 0$) was determined. ●, plus NEM, $J_{max} = 2.62$ μmol/min per mg; ○, control, $J_{max} = 2.55$ μmol/min per mg. (B) J/J_{max} is plotted versus pH. ●, plus NEM, $pIC_{50} = 7.36$, Hill slope = 1.08. ○, control, $pIC_{50} = 7.79$, Hill slope = 1.11. These values were obtained using all the data except for those obtained at the three lowest pH values which deviate from an otherwise linear Hill plot. The assay media and pretreatment procedure are described under Materials and Methods.

$[H^+]$. This relationship is essentially linear for pH values above 7.5, and permits determination of J_{max} by extrapolation to $[H^+] = 0$. The results contained in Fig. 3A show data obtained with normal and NEM-treated mitochondria. NEM has essentially no effect on the value of J_{max}, but the slopes differ by a factor of 2.5 indicating that the proton binding constant has changed. Fig. 3B shows J/J_{max} plotted versus pH for the whole pH range studied. These data show that Cl^- uniport in NEM-treated mitochondria remains fully sensitive to inhibition by protons, suggesting that NEM has not opened a new Cl^- transport pathway. The stimulation observed can be simply explained by an NEM-induced decrease in the pIC_{50} for protons. Thus, the effect induced by NEM is very similar to that induced by pretreatment with mersalyl when transport is assayed in media containing thioglycolate to reverse mersalyl's inhibitory effect [27].

With both control and NEM-treated mitochondria the data deviate from the theoretical curves (linear Hill Plot) at low pH (Fig. 3B). This deviation is not fully understood; however, it may be related to the lag or acceleration in the light scattering traces which is observed at low pH values. Although treatment with NEM decreases the lag (see Fig. 1A), this effect appears to be secondary to the stimulation, since the lag increases as the pH decreases in both control and NEM-treated mitochondria.

Effect of mersalyl on anion uniport in NEM-treated mitochondria

To determine whether NEM reacts at the same sites as mersalyl, the effect of mersalyl on malonate and Cl^- uniport in NEM-treated mitochondria was investigated. Cl^- transport assayed at pH 7.4 (square symbols in Fig. 4A) is inhibited when the dose of mersalyl is increased from about 5 to 7 nmol/mg; however, as observed previously [27], inhibition is only partial reaching a maximum of about 56%. Malonate uniport, on the other hand, is inhibited almost completely over the same dose range suggesting that the extent of inhibition may be related to the size of the anion (see Discussion). Moreover, the NEM-induced malonate flux observed at pH 6.9 is also inhibited by mersalyl. These results indicate that NEM does not react at the inhibitory mersalyl binding site (site 2). The reason for this is unknown; however, other carriers such as the dicarboxylate, tricarboxylate and oxoglutarate carriers are inhibited by mersalyl but insensitive to NEM [28]. The data in Fig. 4A also show that the dose of mersalyl required to inhibit anion uniport in NEM-treated mitochondria is about one third of that required to inhibit transport in normal mitochondria (compare open and closed circles). This result is not unexpected since NEM will have reacted with many of the mersalyl binding sites in the inner membrane.

To determine whether NEM induces the shift in the pH profile by reacting with mersalyl-binding site 1, I looked for stimulation of swelling by mersalyl in NEM-treated mitochondria. The data in Fig. 4A show that, unlike in normal mitochondria (see Fig. 5, Ref. 27) mersalyl does not stimulate Cl^- transport at pH 7.4. Furthermore, the data in Fig. 4B show that pretreatment of NEM-treated mitochondria with mersalyl has no effect on malonate transport (assayed in the presence of 1 mM cysteine to reverse mersalyl's inhibitory

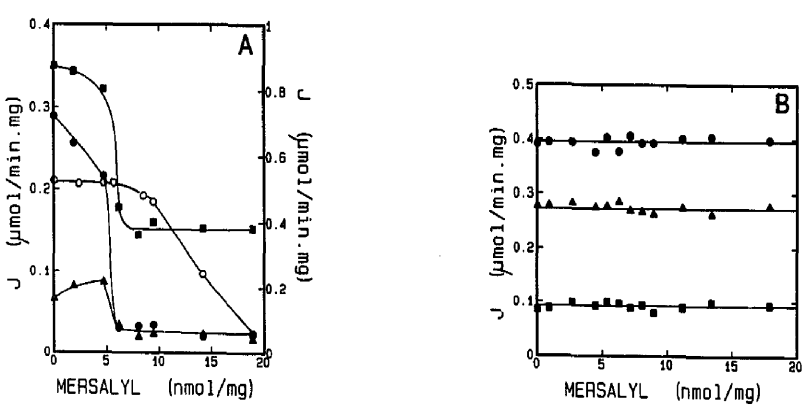

Fig. 4. Effect of mersalyl on anion uniport in NEM-treated mitochondria. The rate of anion uniport is plotted versus the dose of mersalyl. Mitochondria were pretreated with NEM (50 nmol/mg) as described under Materials and Methods except that no cysteine was added. After 10 min, various doses of mersalyl were added to aliquots of the NEM-treated mitochondria and then Cl^- or malonate uniport was assayed using the light scattering technique. (A) ●, ▲, malonate uniport assayed at pH 7.4 and pH 6.9, respectively. ○, malonate uniport assayed at pH 7.4 in control mitochondria (no NEM). ■, Cl^- uniport assayed at pH 7.4 in NEM-treated mitochondria, plotted on the right-hand scale. (B) Results obtained when malonate uniport was assayed with cysteine (1 mM) included in the assay medium to reverse inhibition of transport by mersalyl; ●, pH 8.4; ▲, pH 7.4; ■, pH 6.9. The assay media contained valinomycin (0.5 nmol/mg), nigericin (1 nmol/mg), A23187 (10 nmol/mg) and rotenone (2 μg/mg). See Materials and Methods for further details.

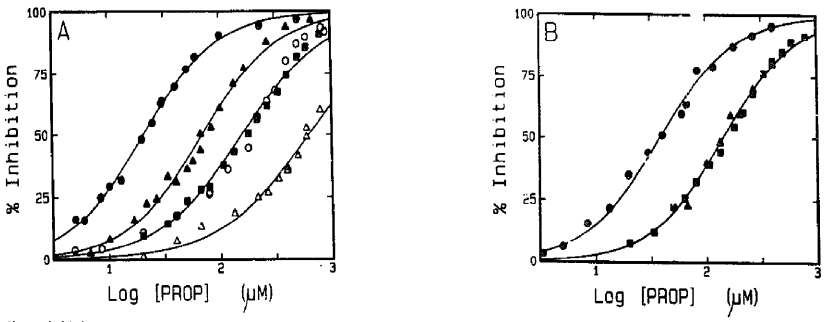

Fig. 5. Effect of thiol group modification on the inhibition of anion uniport by propranolol. Dose-response curves for inhibition of anion uniport by propranolol (PROP) are shown. ●, control; ▲, NEM-pretreated (50 nmol/mg) mitochondria; ○, *p*-CMS-pretreated (22 nmol/mg) mitochondria; △, mersalyl-pretreated (21.6 nmol/mg) mitochondria; ■, mersalyl-pretreated (25 nmol/mg) mitochondria assayed with 1 mM cysteine in the assay. (A) Inhibition of Cl^- uniport. IC_{50} values (μM) for the curves shown are 20.5, 69, 157, and 628 and the corresponding Hill slopes are 1.30, 1.27, 1.14 and 1.06, respectively. (B) Inhibition of malonate uniport. IC_{50} values (μM) for the curves shown are 38 and 141 and the corresponding Hill slopes are 1.28 and 1.32, respectively. The KCl and K malonate assay media contained valinomycin (0.5 nmol/mg), A23187 (10 nmol/mg), nigericin (1 nmol/mg) and rotenone (2 μg/mg). For further details of assays and media see Materials and Methods.

effect) at pH 6.9, 7.4 or 8.4. This lack of effect not only suggests that NEM reacts with the mersalyl-sensitive site (site 1), but also provides further evidence that the stimulation induced by mersalyl is not a non-specific change in membrane permeability.

Modification of 'site 1' increases the IC_{50} for propranolol

The findings that Cl^- transport in mersalyl- and NEM-treated mitochondria is electrophoretic, inhibited by matrix divalent cations, inhibited by protons and inhibited by DCCD led to the conclusion that the transport was mediated by IMAC [27]. It was surprising, therefore, when preliminary studies suggested that propranolol, which blocks Cl^- uniport in normal mitochondria with an IC_{50} of 25 μM [8], was unable to inhibit Cl^- uniport in mersalyl-treated mitochondria. The explanation for this apparent anomalous behavior is revealed by the propranolol dose-response curves contained in Fig. 5A. Full inhibition appears to be possible in normal, NEM-, *p*-CMS- and mersalyl-pretreated mitochondria; however, all these treatments increase the IC_{50} for propranolol. Mersalyl has the largest effect increasing the IC_{50} from 20 μM to 157 μM and 600 μM, when transport is assayed in the presence and absence of cysteine, respectively. This suggests that binding of mersalyl to the inhibitory site may also influence propranolol binding.

The results contained in Fig. 5B show that when malonate transport is assayed, a similar shift is observed indicating that this effect is not peculiar to Cl^-. This finding confirms the conclusion that the change in IC_{50} represents a change in the properties of IMAC and does

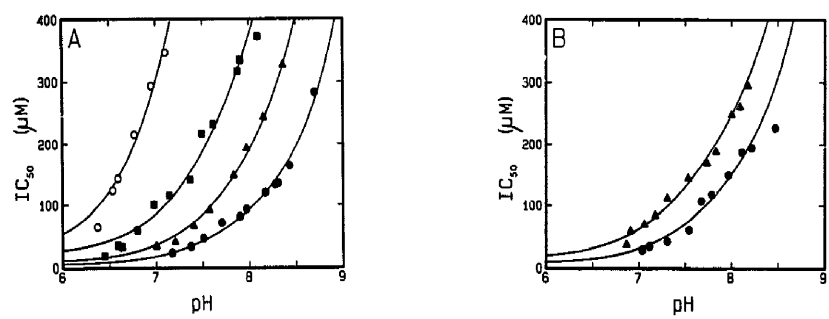

Fig. 6. Effect of thiol group modification on pH dependence of propranolol IC_{50} for inhibition of anion uniport. (A) IC_{50} values for inhibition of Cl^- uniport are plotted versus the pH of the assay medium. ●, control mitochondria; ▲, NEM-pretreated (50 nmol/mg) mitochondria; ■, mersalyl-pretreated (25 nmol/mg) mitochondria assayed with 1 mM cysteine in the assay medium; ○, mersalyl-pretreated (25 nmol/mg) mitochondria. (B) IC_{50} values for inhibition of malonate uniport. ●, control mitochondria; ▲, mersalyl-pretreated (25 nmol/mg) mitochondria assayed with 1 mM cysteine in the assay medium. The IC_{50} values were determined as described in Fig. 5. For composition of assay media and pretreatment procedures see Materials and Methods.

not represent inhibition of a second Cl^- channel with different IC_{50}. With malonate the effects of NEM and mersalyl (plus cysteine in the assay to reverse inhibition by mersalyl) were about the same. Both increased the IC_{50} for propranolol from about 38 μM to 141 μM.

The effect of thiol reactive agents on the pH dependence of the IC_{50} for propranolol

One of the characteristics of inhibition of anion uniport by propranolol and other cationic amphiphiles is that the IC_{50} is dependent on pH [8]. It was possible, therefore, that the change in IC_{50} induced by thiol reactive agents could reflect the elimination of the pH-dependence of the IC_{50}. The data contained in Fig. 6 show that this is not the case. The IC_{50} for Cl^- transport (Fig. 6A) remains dependent on pH in NEM-treated mitochondria, mersalyl-treated mitochondria and mersalyl-treated mitochondria assayed in presence of cysteine. Similar results were obtained when malonate transport was assayed (see Fig. 6B). The pH dependence of the IC_{50} for propranolol has been described in terms of the pK values of two protonation sites and two pK values for the propranolol binding site [8]. It can also be explained, however, in terms of two propranolol binding sites at least one of which requires IMAC to be protonated before propranolol can bind. In both models, however, the pK of the protonation site must be between 6 and 7 and the two binding constants for propranolol must be about 5 μM and 100 μM, respectively [8]. It is difficult to determine which of these values has changed following reaction with the thiol reagents. Most of the data can be fitted to these models by assuming that either the pK of the protonation site or the propranolol binding constants have changed; however, the data obtained with mersalyl alone only fit these models if both these values are changed (analysis not shown).

Modification of 'site 1' increases the IC_{50} for Mg^{2+}

In view of the fact that protonation of matrix sites, possibly a single site, appears to modulate the binding of both propranolol [8] and Mg^{2+} [7], and the finding that the pK of this site may be modulated by pretreatment with NEM and mersalyl (Fig. 6), I also investigated the effect of these reagents on the inhibition of anion uniport by matrix Mg^{2+}. The data contained in Fig. 7 show the results of an experiment in which inhibition in normal and NEM-treated mitochondria was compared. In control mitochondria, as previously demonstrated [7], in the presence of A23187 the IC_{50} is about 37 μM. In the NEM-treated mitochondria the value is about 86 μM more than 2-fold higher. Note that the data are fully consistent with complete inhibition of anion uniport by Mg^{2+} under both conditions. A similar effect was induced by mersalyl (results not shown).

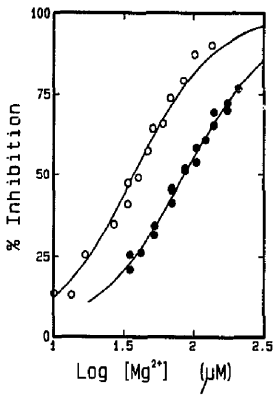

Fig. 7. Effect of thiol group modification on the inhibition of Cl^- uniport by Mg^{2+}. Dose response curves for inhibition of Cl^- uniport by Mg^{2+} are shown for control (●) and NEM-pretreated (50 nmol/mg) (○) mitochondria. Cl^- uniport was assayed using the light scattering technique in KCl assay medium containing nigericin (1 nmol/mg), A23187 (10 nmol/mg), rotenone (2 μg/mg) and various doses of Mg^{2+}. Net transport of KCl was initiated by the addition of valinomycin (0.5 nmol/mg) 0.2 min after addition of the mitochondria (0.1 mg/ml) to the assay medium. Mg^{2+} concentration was varied and the concentration in the medium is plotted. The IC_{50} values obtained were 37.2 μM (Hill slope = 1.5) and 85.6 μM (Hill slope = 1.36) for the control and NEM-treated mitochondria, respectively. See Materials and Methods for further details.

The effect of NEM on pH dependence of the IC_{50} for Mg^{2+}

Since the Mg^{2+} IC_{50} in normal mitochondria is found to be a function of pH [7], I also investigated whether the change in IC_{50} induced by NEM might reflect the elimination of the pH dependence. Thus, the IC_{50} for Mg^{2+} was determined in NEM-treated and

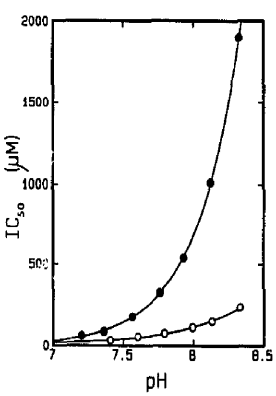

Fig. 8. Effect of thiol group modification on the pH dependence of the Mg^{2+} IC_{50} for Inhibition of Cl^- uniport. The IC_{50} values are plotted versus the pH of the assay medium. ○, control mitochondria; ●, NEM-pretreated (50 nmol/mg) mitochondria. The IC_{50} values were determined as described in Fig. 7 except the pH of the assay medium was varied. See Materials and Methods for further details.

control mitochondria in media at various pH values. The results, contained in Fig. 8, reveal that the IC_{50} remains pH dependent, increasing from a value of 60 μM at pH 7.2 up to 1.9 mM at pH 8.3. Whereas the data obtained with normal mitochondria yield a linear relation between IC_{50} and $1/[H^+]$, consistent with Mg^{2+} binding being dependent on the binding of a single proton (see Ref. [7]) the data obtained with NEM-treated mitochondria yield a linear relation between IC_{50} and $1/[H^+]^{1.36}$. Thus in NEM-treated mitochondria the IC_{50} is more sensitive to pH than in normal mitochondria. From these data it is not possible to say whether the intrinsic binding constant for Mg^{2+} has changed. The rates of transport become so low at low pH that it becomes very difficult to determine an accurate value for the IC_{50}.

Previously, I have shown that external Mg^{2+} is also able to inhibit anion uniport but with a much higher IC_{50} [7]. This IC_{50} also appears to be increased by treatment with NEM (results not shown), however, because the values are so high it becomes difficult to distinguish true inhibition from osmotic effects.

Discussion

In this paper, I have presented evidence that the thiol alkylating agent NEM and the mercurials mersalyl and *p*-CMS react with IMAC at a common site and have a profound effect on the modulation of IMAC by protons, propranolol and Mg^{2+}. These findings underscore the complexity of the regulation of this unique transport pathway. The cartoon contained in Fig. 9 is introduced to provide a summary of the relationships between these effects and represents our working model of this transport pathway. Thus, Fig. 9A indicates that Mg^{2+} is believed to inhibit transport by binding to two separate sites, one is located on the inner surface of IMAC in the matrix and the other on the outer surface. The IC_{50} values are 38 μM and 450 μM respectively at pH 7.4 [7]. Protons also inhibit from the inside with a pIC_{50} of 7.8 [1]. The exact location of these sites with respect to the substrate binding site or channel opening is unknown.

Transport is also inhibited irreversibly by the hydrophobic alkylating agent *N,N'*-dicyclohexylcarbodiimide [9,10] and reversibly by cationic amphiphiles such as propranolol [8]. Since these drugs are both lipophilic, these sites are probably located in or close to the lipid bilayer. Fig. 9A also shows that there is believed to be a second proton binding site in the matrix with a p*K* between 6 and 7 which plays a part in the binding of matrix Mg^{2+}, external Mg^{2+} [7] and propranolol [8]. This is evidenced by a strong pH dependence of the IC_{50} values for these inhibitors. Propranolol and Mg^{2+} are not believed to bind to a common site for two reasons: (1) other drugs such as clonazepam which are not charged also inhibit [8], and (2) Mg^{2+} and propranolol do not compete with each other. Partial inhibition by Mg^{2+} actually lowers the IC_{50} for propranolol (Beavis, A.D., unpublished data).

The two putative thiol groups which react with mercurials are also thought to be on the outer surface [27]. The evidence for the existence of two binding sites (sites 1 and 2) is presented in Ref. 27. In essence it centers on two observations: [1] Mersalyl pretreatment

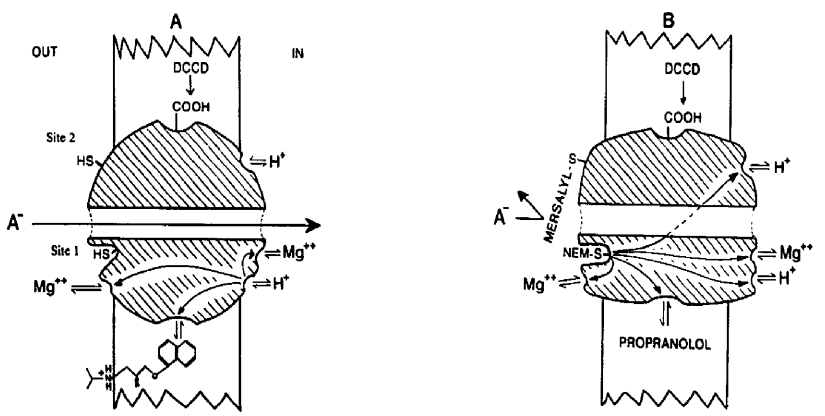

Fig. 9. Hypothetical model summarizing the effect of thiol group modification on the properties of IMAC. (A) IMAC is an anion uniport pathway which is inhibited by reaction with DCCD [9], matrix H^+ [1], matrix Mg^{2+} [1,7], cationic amphiphiles [8] and cytosolic Mg^{2+} [7]. A second H^+ binding site in the matrix increases the IC_{50} for cationic amphiphiles, matrix Mg^{2+} and cytosolic Mg^{2+}. Two thiol groups also exist on the outside, one (site 1) lies in a cleft whereas the other is more exposed and close to the channel mouth or substrate binding site. (B) Reaction of NEM (or mersalyl) at site 1 induces a conformational change which leads to an increase in IC_{50} for matrix H^+, matrix Mg^{2+}, cationic amphiphiles and cytosolic Mg^{2+}. It may also decrease the affinity of the regulatory protonation site. It does not appear to affect reaction with DCCD. Once reacted this thiol is inaccessible to aqueous solutes and binding of both mersalyl and NEM is irreversible. Site 2 reacts with mersalyl but not NEM and due to a steric effect inhibits the transport of most anions. Small anions, however, can still be transported but at a lower rate.

can stimulate transport at low pH but not at high pH, (i.e., the pH profile is shifted), when uniport is assayed in the presence of cysteine or thioglycolate (site 1 effect). [2] Mersalyl inhibits transport when assayed in the absence of excess aqueous thiols (site 2 effect). In the present paper, it is shown that NEM not only induces an effect which is similar to that induced by mersalyl at 'site 1', but that it also totally blocks any further stimulation of anion uniport by mersalyl. Thus, NEM and mersalyl probably bind to the same site. The arrows in Fig. 9B are used to indicate that binding of NEM, mersalyl and *p*-CMS to 'site 1' increases the IC_{50} for inhibition by protons, matrix Mg^{2+}, external Mg^{2+} and propranolol. There may also be a decrease in the p*K* of the modulatory protonation site in the matrix, since changes in the p*K* of this group are hard to distinguish from changes in the intrinsic binding constants for Mg^{2+} and propranolol. In view of the suggested relationship between IMAC and the uncoupling protein [11], it is interesting to note that NEM has also been reported to increase the IC_{50} for inhibition of the uncoupling protein by GDP [22,24,25] and that it stimulates H^+ and Cl^- fluxes through the protein [22,25]. The effect of NEM is, therefore, reminiscent of the effect of pH. In both IMAC and the uncoupling protein pH regulates the binding of a major physiological inhibitor, Mg^{2+} [7] and purine nucleotides [21,22], respectively.

Since the effects of site 1 modification are so diverse, it is possible that the interaction of the ligand at this site causes a large conformational change in the protein as indicated in Fig. 9. To explain the irreversibility of the site 1 effects induced by mersalyl, it is suggested that site 1 may lie in a cleft. An interaction between the R-group and the protein could then be involved in the conformational change. Effects of this interaction could also explain why the various reagents do not necessarily affect the other interactions to the same extent (e.g. see Figs. 6A and 7A). I have looked for an effect of NEM on the first order kinetics of inhibition by *N,N'*-dicyclohexylcarbodiimide but there does not appear to be one (results not shown) and, consequently, no interaction between these sites is indicated.

Reaction of mersalyl with the second putative thiol group ('site 2') inhibits anion uniport. However, unlike Mg^{2+}, H^+, propranolol and DCCD, mersalyl does not inhibit completely the transport of all anions [27]. Because of this finding, this site is depicted as lying near but not in the channel opening (or substrate binding site). It is suggested that inhibition may result from a steric effect in which the large anionic organic side chain of mersalyl blocks the channel opening preventing large multivalent anions from entering but permitting small monovalent anions to be transported albeit at a diminished rate. This conclusion is supported by the finding that *p*-CMS which has a smaller organic R-group has virtually no effect on Cl^- transport and incompletely inhibits malonate transport [27]. Since inhibition is readily reversed by thiols this sulfhydryl group is shown to be accessible to the aqueous phase. Furthermore, binding of mercurials to this site per se does not appear to affect the conformation of the channel, e.g., *p*-CMS reacts with this site but has no effect on the pH dependence of Cl^- transport [27]. Again, it is interesting to note a similarity between IMAC and the uncoupling protein. Just as the extent of inhibition of transport via IMAC by mersalyl is dependent on the anion studied, it has been reported that the extent of inhibition of H^+ and Cl^- transport via the uncoupling protein by mersalyl also differs [23,24].

The finding that NEM has no effect on the rate of uniport at high pH not only suggests that NEM does not bind to site 2, but also indicates that the reaction at site 1 per se has no direct effect on transport. Lack of reactivity with site 2 is confirmed by the finding that mersalyl can still inhibit uniport in NEM-pretreated mitochondria (Fig. 4). These findings provide further evidence for the existence of the two functionally important thiol groups. Since NEM itself has no effect on transport rates, it is ideal reagent for examining further the effects mediated via 'site 1'.

In freshly isolated mitochondria the activity of the anion uniporter is very low. This is chiefly a consequence of inhibition by matrix Mg^{2+}. Activity is revealed either by depleting endogenous Mg^{2+} [1] or by raising the matrix pH, which both increase the activity of IMAC and increase the IC_{50} for Mg^{2+} [7]. Previously [7], we have argued that this regulatory mechanism should make anion uniport very sensitive to small changes in matrix pH and that this could be a physiological regulatory mechanism. Selwyn's group [12] has suggested that palmitoyl-CoA may also be a physiological inhibitor of this pathway, presumably by binding to the outer surface of IMAC since this compound cannot enter the matrix. In this paper, I have now shown that there may be another important regulatory site on the outer surface of IMAC. Although mercurials and NEM are not physiological agents they may mimic the effect of a physiological regulator. Thus, any agent binding at or near the NEM-reactive site could have two effects. First, inhibition by Mg^{2+} would be decreased and second, inhibition by matrix protons would be decreased. Thus, there could be a significant stimulation of anion uniport. From the data in Fig. 8 it can be seen that at pH 8.0 (matrix pH $\approx$ 7.8) treatment with NEM raises the Mg^{2+} IC_{50} from 112 μM to 680 μM. This could lead to a significant stimulation of anion uniport since in the cell the concentration of free Mg^{2+} in the matrix is believed to be about 0.37 mM [34]. Furthermore, from Fig. 8 it is also evident that any simultaneous increase in matrix pH would amplify the effect considerably.

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