# The ATP-Binding Site of the Human Placental $H^+$ Pump Contains Essential Tyrosyl Residues†

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ABSTRACT: Transient exposure of human placental brush-border membrane vesicles to cholate reorients the ATP-driven $H^+$ pump, enabling the pump to transport $H^+$ into the vesicles upon addition of ATP to the external medium. $H^+$ uptake can be measured in these vesicles by following the decrease in the absorbance of acridine orange, a $\Delta$pH indicator. We investigated the role of tyrosyl residues in the catalytic function of the $H^+$ pump by studying the effects of tyrosyl group specific reagents on ATP-driven $H^+$ uptake in cholate-pretreated membrane vesicles. The reagents tested were 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl), *N*-acetylimidazole, tetranitromethane, and *p*-nitrobenzenesulfonyl fluoride. Treatment of the membrane vesicles with these reagents resulted in the inhibition of the ATP-driven $H^+$ uptake, and the inhibitory potency was in the following order: NBD-Cl > tetranitromethane > *p*-nitrobenzenesulfonyl fluoride > *N*-acetylimidazole. The inhibition of the $H^+$ pump by NBD-Cl was reversible by 2-mercaptoethanol, and the inhibition by *N*-acetylimidazole was reversible by hydroxylamine. Since these reagents are not absolutely specific for tyrosyl groups and can also react with thiol groups, we studied the interaction of *N*-acetylimidazole with the $H^+$ pump whose triol groups were masked by reaction with *p*-(chloromercuri)benzenesulfonate. The SH-masked pump was totally inactive, but the activity could be restored by dithiothreitol. On the contrary, the activity of the SH-masked $H^+$ pump which was subsequently treated with *N*-acetylimidazole could not be restored by dithiothreitol, suggesting that thiol groups were not involved in the inhibition of the $H^+$ pump by *N*-acetylimidazole. The $H^+$ pump was protected from the *N*-acetylimidazole-induced inhibition by its substrate, ATP. ADP, a reversible inhibitor of the pump, also offered protection, whereas adenosine and adenine did not. The protection with ATP was demonstrable not only in the case of *N*-acetylimidazole but also in the cases of NBD-Cl, tetranitromethane, and *p*-nitrobenzenesulfonyl fluoride. Kinetic analysis of the ATP-driven $H^+$ pump in control and *N*-acetylimidazole-treated vesicles revealed that the inhibition of the pump by *N*-acetylimidazole was due to a decrease in the affinity of the pump for ATP as well as a decrease in its maximal velocity. These results strongly suggest that the human placental $H^+$ pump contains tyrosyl residues that are critical for its catalytic activity and that the location of these essential tyrosyl residues and the ATP-binding site interact with each other sterically and/or allosterically.

There are three classes of $H^+$-ATPases ($H^+$ pumps), namely, the P type ($E_1$–$E_2$ type), the F type ($F_0$–$F_1$ type), and the V type (vacuolar type), which can be differentiated from each other by means of their sensitivity to various inhibitors [for a review, see Forgac (1989) and Nelson and Taiz (1989)]. Two inhibitors, *N*-ethylmaleimide (NEM)[1] and 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl), have been extensively used to characterize the V-type $H^+$ pump because this type is extremely sensitive to inhibition by these reagents compared to the other two types. Both NEM and NBD-Cl are capable of causing chemical modification of certain specific amino acid residues in proteins. NEM is relatively specific for cysteinyl residues (thiol groups) whereas NBD-Cl can react with cysteinyl (thiol group) and lysyl (amino group) as well as tyrosyl (phenolic hydroxyl group) residues. Inhibition of the V-type $H^+$ pump by these two reagents is protectable by ATP (Arai et al., 1987; Bowman et al., 1986; Forgac & Cantley, 1984; Mandala & Taiz, 1986; Randall & Sze, 1987; Uchida et al., 1988; Wang & Sze, 1985), indicating that the reactive amino acid residues are located at or near the ATP-binding site.

Because of the selectivity of the NEM reaction, inhibition of the V-type $H^+$ pump by NEM can be taken as evidence that cysteine is the NEM-reactive amino acid residue which is essential for the activity of the pump. The observation that the NEM-induced inhibition is reversible with high concentrations of free thiols such as dithiothreitol (DTT) (Wang & Sze, 1985) provides further support for the involvement of cysteinyl residues in the NEM reaction. On the other hand, NBD-Cl potentially can react with cysteinyl, tyrosyl, and lysyl residues, and any one of them may represent the NBD-Cl-

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[1] Abbreviations: NEM, *N*-ethylmaleimide; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; DTT, dithiothreitol; pCMBS, *p*-(chloromercuri)benzenesulfonic acid; pNBSF, *p*-nitrobenzenesulfonyl fluoride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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reactive amino acid residue in the $H^+$ pump. Inhibition by NBD-Cl has been shown to be reversible with free thiols (Forgac & Cantley, 1984; Randall & Sze, 1987; Uchida et al., 1988), and this characteristic renders the lysyl residue an unlikely candidate for NBD-Cl reaction. This leaves tyrosyl and cysteinyl residues as the potential sites for chemical modification by NBD-Cl. While the involvement of cysteinyl residues has been probed with additional reagents such as NEM and *p*-(chloromercuri)benzenesulfonate (pCMBS), the possible participation of tyrosyl residues in the catalytic activity of the $H^+$ pump has not received much attention. To our knowledge, the only study which has provided evidence for the inhibition of a V-type $H^+$ pump by chemical modification of tyrosyl residues is that of Uchida et al. (1988). These investigators, working with the $H^+$ pump present in the vacuolar membrane of *Saccharomyces cerevisiae*, have shown that NBD-Cl inhibits the activity of the pump by reacting with a single tyrosyl residue.

Recently, we have obtained evidence for the existence of an ATP-driven $H^+$ pump in human placental brush-border membranes, and the properties of the pump indicate that it belongs to the class of V-type $H^+$ pumps (Simon et al., 1990). The placental pump is inhibited by micromolar concentrations of NEM in an ATP-protectable manner. These results can be taken as evidence for the presence of cysteinyl residue(s) at or near the ATP-binding site. In the present paper, we have investigated the possible role of tyrosyl residues in the catalytic function of the $H^+$ pump. This study was conducted by using not only NBD-Cl but also additional tyrosyl group specific reagents such as tetranitromethane, *p*-nitrobenzenesulfonyl fluoride (pNBSF), and *N*-acetylimidazole as probes. Treatment of human placental brush-border membranes with these reagents leads to inactivation of the $H^+$ pump. Furthermore, the pump can be protected by ATP from this inactivation. These results strongly suggest that the human placental $H^+$ pump contains tyrosyl residue(s) which is (are) critical for its catalytic activity and that these residue(s) is (are) located at or near the ATP-binding site.

## Materials and Methods

Sodium cholate, acridine orange, valinomycin, ATP, NBD-Cl, *N*-acetylimidazole, pNBSF, pCMBS, hydroxylamine, 2-mercaptoethanol, DTT, and Sephadex G-50 (bead size, 20–80 μm) were purchased from Sigma Chemical Co., St. Louis, MO. Tetranitromethane was obtained from Aldrich Chemical Co., Milwaukee, WI. Polystyrene disposable cuvettes were obtained from Sarstedt, Newton, NC. All other chemicals were of analytical grade.

*Preparation of Human Placental Brush-Border Membrane Vesicles.* Brush-border membrane vesicles were prepared from human term placentas as previously described (Ganapathy et al., 1985; Balkovetz et al., 1986) using a $Mg^{2+}$ aggregation method. Only normal placentas which were obtained within 2 h of delivery were used. All the steps involved in the isolation procedure were carried out at 4 °C. Briefly, the villous tissue was collected after removal of decidua and chorionic plate. The tissue was cut into small pieces and washed with an isotonic buffer [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)/tris(hydroxymethyl)aminomethane (Tris), pH 7.0, containing 300 mM mannitol] in order to remove blood. The washed tissue fragments were then agitated in 300 mL of the same buffer for 30 min on a magnetic stirrer. This procedure resulted in the release of brush-border membranes from syncytiotrophoblast. The slurry was then filtered through three layers of cheesecloth, and the resulting filtrate was centrifuged at 8000*g* for 15 min. The supernatant containing brush-border membranes was again centrifuged at 73000*g* for 30 min. The pellets representing crude brush-border membranes were suspended in 50 mL of buffer using 10 strokes with a Dounce homogenizer (loose-fitting pestle). A stock solution of 1 M $MgCl_2$ was added to the homogenate to give a final concentration of 12 mM $MgCl_2$. The mixture was stirred for 1 min and let stand for 10 min. It was then centrifuged at 3000*g* for 15 min to remove $Mg^{2+}$-aggregated non-brush-border membranes. The supernatant containing brush-border membranes was centrifuged at 73000*g* for 30 min. The pellets were suspended in 5 mM Hepes/Tris buffer (pH 7.4) containing 150 mM KCl and centrifuged again. The resulting pellets were finally suspended in a small volume of the above buffer. The protein concentration of the suspension was adjusted to 10 mg/mL and stored in liquid $N_2$ in small aliquots. The purity of the brush-border membranes was occasionally checked by determining the activity of alkaline phosphatase, a brush-border marker enzyme. The enrichment of the activity in membrane preparations (compared to the activity in the original homogenate of the washed placental tissue) normally varied within a range of 20–30, with an average value of 23 ± 3 ($n$ = 4).

Protein was determined according to the method of Lowry et al. (1951) using bovine serum albumin as the standard. Alkaline phosphatase activity was determined with *p*-nitrophenyl phosphate as the chromogenic substrate (Ganapathy et al., 1986).

*Detergent Treatment and Reorientation of the $H^+$ Pump.* Cholate-induced reorientation of the $H^+$ pump in human placental brush-border membrane vesicles was carried out according to the procedure described by Simon and Burckhardt (1990). On the day of the experiment, the frozen brush-border membrane vesicles were thawed at 37 °C, cooled down again on ice, and diluted with 5 mM Hepes/Tris buffer (pH 7.4) containing 150 mM KCl to a protein concentration of 3.7 mg/mL. A stock solution of 10% sodium cholate made in water was added to the membrane suspension dropwise with frequent mixing of the detergent–membrane mixture. The final concentration of the detergent was 1%, and that of protein was 3.3 mg/mL. After the mixture was kept on ice for 3 min, it was passed through a Sephadex G-50 column in order to remove the detergent. The column was equilibrated with 5 mM Hepes/Tris buffer (pH 7.4) containing 150 mM KCl prior to the application of the sample. For a 5-mg protein sample, the size of the column was normally 1 cm × 15 cm. The turbid fractions containing the re-formed vesicles were collected and used for measurement of the $H^+$ pump activity. The protein concentration in these fractions was always within the range of 1.5–2.5 mg/mL.

*Treatment with Tyrosyl Group Specific Reagents.* (*A*) *NBD-Cl.* Cholate-pretreated membrane vesicles were incubated in the cuvette in the presence or absence of NBD-Cl of desired concentration at room temperature for 15 min. A stock solution of NBD-Cl was made in ethanol. Control vesicles received ethanol alone. The cuvette buffer contained 50 mM Hepes/Tris (pH 7.4), 150 mM KCl, 5 mM $MgCl_2$, 10 μM valinomycin, and 6 μM acridine orange. Following incubation, $H^+$ uptake was initiated by addition of 2 mM ATP.

(*B*) *N-Acetylimidazole.* Cholate-pretreated membrane vesicles were incubated in the cuvette in the presence or absence of *N*-acetylimidazole of the desired concentration at room temperature for 30 min. A stock solution of *N*-acetylimidazole was prepared in a pH 7.4 buffer. Following incubation, 1 mL of cuvette buffer containing 50 mM Hepes/Tris (pH 7.4), 150 mM KCl, 5 mM $MgCl_2$, 10 μM

valinomycin, and 6 μM acridine orange was added. $H^+$ uptake was initiated by addition of 2 mM ATP.

(*C*) *Tetranitromethane and pNBSF.* Incubation of cholate-pretreated membrane vesicles with tetranitromethane or pNBSF was done in 1 mL of cuvette buffer containing 50 mM Hepes/Tris (pH 8.0), 150 mM KCl, 5 mM $MgCl_2$, 10 μM valinomycin, and 6 μM acridine orange. Treatment was carried out at room temperature for 15 min. Stock solutions of inhibitors were prepared in ethanol, and control vesicles received an equal amount of ethanol alone. Following incubation, $H^+$ uptake was initiated by addition of 2 mM ATP.

*Reversal of Inhibition.* Reversal of NBD-Cl-induced inhibition of the placental $H^+$ pump by 2-mercaptoethanol was investigated as follows. Cholate-pretreated membrane vesicles were incubated with or without 20 μM NBD-Cl for 15 min at room temperature. Unreacted NBD-Cl was removed by passing the mixture through a Sephadex G-50 column. The control and treated eluates were divided into two parts. One part was incubated with 10 mM 2-mercaptoethanol prepared in 5 mM Hepes/Tris in 150 mM KCl, pH 7.4, and the other with the buffer alone for 30 min at room temperature. The vesicles were then passed through a Sephadex G-50 column, and the eluted membrane vesicles were used for measurement of the $H^+$-pump activity.

Reversal of *N*-acetylimidazole-induced inhibition by hydroxylamine was investigated as follows. Cholate-pretreated membrane vesicles were incubated with or without 2 mM *N*-acetylimidazole for 30 min at room temperature. Unreacted reagent was removed by passing the mixture through a Sephadex G-50 column. The control and treated eluates were divided into two parts. One part was incubated with 150 mM KCl in 5 mM Hepes/Tris buffer, pH 7.4, and the other with 75 mM hydroxylamine hydrochloride plus 75 mM KCl in 5 mM Hepes/Tris buffer, pH 7.4. Incubation was done at 37 °C for 30 min. The vesicles were then passed through a Sephadex G-50 column to remove the reagents, and the eluted vesicles were used for $H^+$-uptake measurements.

*Interaction of N-Acetylimidazole with the SH-Masked $H^+$ Pump.* Cholate-pretreated membrane vesicles were incubated with pCMBS in 5 mM Hepes/Tris buffer (pH 7.4) containing 150 mM KCl for 15 min at room temperature. Unreacted pCMBS was removed by filtration through a Sephadex G-50 column. The eluted vesicles were incubated in the presence or absence of *N*-acetylimidazole for 30 min at room temperature following which the gel filtration step was repeated to remove the unreacted reagent. Incubation of the resultant vesicles with DTT was done for 15 min at room temperature. The vesicles were again subjected to gel filtration prior to measurement of the $H^+$-pump activity.

*Substrate Protection.* Cholate-pretreated membrane vesicles were incubated with or without group-specific reagent at room temperature in the presence or absence of protecting compounds. $MgCl_2$ (5 mM) was present in all cases. Following incubation, the mixtures were passed through a Sephadex G-50 column to remove the reagents, and the eluted vesicles were used for $H^+$-uptake measurements.

*Determination of the Activity of the ATP-Driven $H^+$ Pump.* ATP-driven $H^+$-pump activity was assayed by measuring the uptake of $H^+$ into the vesicles upon addition of ATP to the external medium. $H^+$ uptake was determined by monitoring intravesicular acidification resulting from $H^+$ influx, and this was done by following the absorbance decrease of the ΔpH indicator, acridine orange, as described by Sabolic et al. (1985). Briefly, vesicles (150–250 μg of protein) were added to a cuvette containing 1 mL of 50 mM Hepes/Tris, 150 mM KCl, 5 mM $MgCl_2$, 10 μM valinomycin, and 6 μM acridine orange. When the absorbance of the dye as measured by dual-wavelength photometry (492–540 nm) with an Aminco DW-2 dual-wavelength spectrophotometer had stabilized, 20 μL of 100 mM ATP solution, whose pH had been adjusted to 7.4 with Tris, was added. This resulted in a final ATP concentration of 2 mM in the cuvette. Following the addition of ATP, the decrease in the absorbance with time was recorded. Initial rates of the $H^+$-pump activity were calculated from the slopes of the curves recording the absorbance decrease with time.

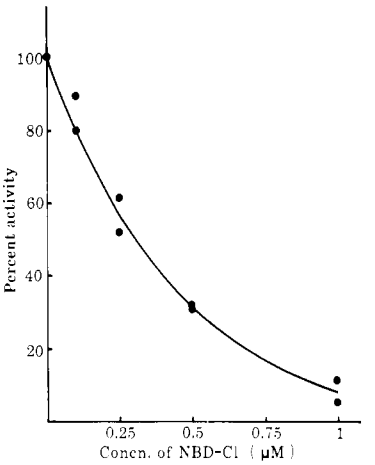


FIGURE 1: Dose–response of NBD-Cl-induced inhibition of the $H^+$ pump. Cholate-pretreated membrane vesicles were incubated with increasing concentrations of NBD-Cl (range, 0–1 μM) (for details, see Materials and Methods) following which ATP-driven $H^+$ uptake was measured by monitoring the absorbance decrease of acridine orange. The results are from a single experiment done in duplicate.

*Statistics.* Duplicate or triplicate determinations were made for each experimental condition. The results from two to three different membrane preparations were pooled and are given as means ± SE. When original drawings are given, each is a representative of the experiment done in replicates. The $H^+$-pump activity, expressed as $\Delta A$ per milligram per minute, varied between different membrane preparations. The range was 0.01468–0.07831 $\Delta A$/(mg·min) with an average value of 0.0370 ± 0.0038 $\Delta A$/(mg·min) (seven membrane preparations). Owing to the variability in the activities of the $H^+$ pump in different membrane preparations, the results of some of the experiments are given as percent activity rather than absolute activity.

## Results and Discussion

*Inhibition of the Human Placental $H^+$ Pump by NBD-Cl.* We investigated the inhibition of the human placental $H^+$ pump by NBD-Cl in cholate-pretreated membrane vesicles. Figure 1 describes the effect of NBD-Cl concentration on the activity of the $H^+$ pump. The inhibition of the pump increased with increasing concentration of NBD-Cl, and a 50% inhibition was observed at a NBD-Cl concentration of approximately 0.3 μM.

Many studies have reported the use of NBD-Cl to modify tyrosyl residues in enzymes as well as transport proteins (Ferguson et al., 1974, 1975; Lin et al., 1982; Hsyu & Giacomini, 1987; Kulanthaivel et al., 1989; Ganapathy et al., 1989). However, the reagent can also react with thiol groups (Birkett et al., 1970; Nitta et al., 1979) and amino groups (Ghosh & Whitehouse, 1968; Aboderin et al., 1973). However, the reaction with amino groups occurs at a significant rate only at pH values greater than 8 and is almost negligible at pH 7.0–7.5 (Aboderin et al., 1973). Since the cholate-pretreated vesicles were incubated with NBD-Cl at pH 7.4,

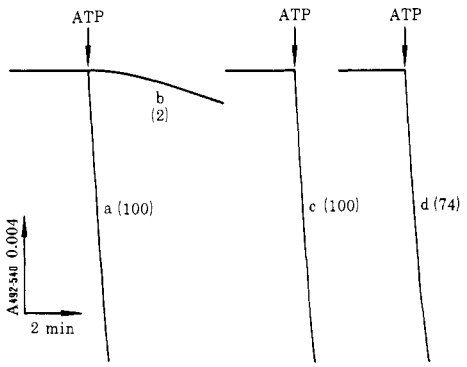


FIGURE 2: Reversal of NBD-Cl-induced inhibition by 2-mercaptoethanol. Cholate-pretreated membrane vesicles were incubated in the presence (curves b and d) or absence (curves a and c) of 20 $\mu$M NBD-Cl for 15 min at room-temperature. Following incubation, the vesicles were passed through a Sephadex G-50 column to remove the unreacted reagent. The eluates were divided into two equal parts and incubated with (curves c and d) or without (curves a and b) 10 mM 2-mercaptoethanol for 30 min at room temperature. The vesicles were again passed through a Sephadex G-50 column to remove the unreacted reagent, and the eluates were used for ATP-driven $H^+$-uptake measurements by monitoring the decrease in the absorbance of acridine orange. The values given in parentheses for curves b and d represent $H^+$-pump activity as the percent of activity in their respective controls, curves a and c. The results are from a representative experiment.

the participation of amino groups in the reaction with NBD-Cl was unlikely. At this pH, the reaction of NBD-Cl with thiol and tyrosyl groups is extensive. In order to show that the inhibition of the placental $H^+$ pump by NBD-Cl was due to the chemical modification of tyrosyl and/or thiol groups, we studied the reversibility of the NBD-Cl inhibition by thiols. It has been demonstrated by other investigators that the phenolic hydroxyl and thiol groups which were modified with NBD-Cl could be regenerated by subsequent reaction with free thiols (Ferguson et al., 1975; Cantley et al., 1978). Therefore, if the inhibition of the $H^+$ pump by NBD-Cl is reversible with thiols, it can be taken as supporting evidence for the involvement of tyrosyl and/or thiol groups in the NBD-Cl reaction. Figure 2 describes an experiment in which the effect of 2-mercaptoethanol on the NBD-Cl-induced inhibition of the placental $H^+$ pump was investigated. In this experiment, cholate-pretreated membrane vesicles were subjected to four different maneuvers in order to provide proper controls for the assessment of the reversibility of the NBD-Cl reaction: control vesicles (a); vesicles treated with 20 $\mu$M NBD-Cl (b); vesicles treated with 10 mM 2-mercaptoethanol (c); and vesicles treated with 20 $\mu$M NBD-Cl followed by treatment with 10 mM 2-mercaptoethanol (d). Finally, the unreacted reagents were removed, and the activity of the $H^+$ pump was measured in these vesicles. Incubation of the vesicles with NBD-Cl caused almost complete inhibition of the $H^+$ pump (curve b vs curve a), but this inhibition was substantially reversed with 2-mercaptoethanol (curve d vs curve c). It is clear from this experiment that the NBD-Cl-induced inhibition of the $H^+$ pump is reversible with thiols, indicating that the reaction with NBD-Cl leads to chemical modification of tyrosyl and/or thiol groups in the human placental $H^+$ pump.

*Inhibition of the Human Placental $H^+$ Pump by Tyrosyl Group Specific Reagents.* The participation of thiol groups in the maintenance of optimal activity of the V-type $H^+$ pumps has been thoroughly studied with NEM as a selective modifier of thiol groups. The goal of the present study was to investigate whether tyrosyl residues are also critical for optimal activity of the V-type $H^+$ pumps. Since the inhibition of the placental $H^+$ pump by NBD-Cl as well as the reversibility of this inhibition by thiols does not distinguish between the participation of tyrosyl groups and that of thiol groups, we investigated the influence of three additional reagents which are commonly used to modify tyrosyl residues in proteins. These reagents are tetranitromethane (Glazer, 1975; Lundblad & Noyes, 1984), pNBSF (Liao et al., 1982), and *N*-acetylimidazole (Cohen, 1968). Incubation of cholate-pretreated membrane vesicles with all three reagents led to inactivation of the $H^+$ pump. Table I compares the inhibitory potencies of these reagents with that of NBD-Cl as assessed from the $K_{0.5}$ values (the inhibitor concentration which produces a 50% inactivation of the $H^+$-pump activity). The inhibitory potency appears to be in the following order: NBD-Cl > tetranitromethane > pNBSF > *N*-acetylimidazole.

Table I: $K_{0.5}$ Values for Inhibition of the Placental $H^+$ Pump by Tyrosyl Group Specific Reagents[a]

| inhibitor | $K_{0.5}$ ($\mu$M) |
|---|---|
| NBD-Cl | 0.18 ± 0.05 |
| tetranitromethane | 3.68 ± 0.19 |
| pNBSF | 27.1 ± 5.2 |
| *N*-acetylimidazole | 404 ± 73 |

[a] A Dixon plot (1/velocity versus inhibitor concentration) was used to determine $K_{0.5}$ values. The $K_{0.5}$ value is defined as the concentration of the inhibitor which causes 50% inactivation of the $H^+$-pump activity. The results, given as means ± SE, are from four determinations done with two membrane preparations.

Even though NBD-Cl, tetranitromethane, pNBSF, and *N*-acetylimidazole have all been used to produce chemical modification of tyrosyl groups in proteins, *N*-acetylimidazole appears to be the most selective among these reagents for the purpose (Cohen, 1968). For example, tetranitromethane can also interact with thiol groups in model compounds such as glutathione (Sokolovsky et al., 1969) as well as in proteins (Riordan & Christen, 1968). In contrast, the reactivity of *N*-acetylimidazole with amino acid residues other than tyrosyl residues is minimal. This reagent can modify thiol groups and amino groups to some extent, but there are means to differentiate between the reaction of *N*-acetylimidazole with thiol, amino, and phenolic hydroxyl groups. The reagent acetylates thiol groups in proteins, but the products immediately hydrolyze in aqueous solutions, regenerating the free thiol groups (Masiak & D'Angelo, 1975). This means that the inhibition of the placental $H^+$ pump by *N*-acetylimidazole in aqueous solutions should be spontaneously reversible if the inhibition is due to modification of thiol groups. However, there was no indication of such a spontaneous reversal of the *N*-acetylimidazole-induced inhibition of the $H^+$ pump in the present study, and hence the participation of thiol groups in the reaction was unlikely. Amino groups and phenolic hydroxyl groups are also acetylated by the reagent. Even though acetylation of both of these groups can be reversed by subsequent treatment with hydroxylamine, these groups can be distinguished by the ease with which the reversal is effected. Hydroxylamine at neutral pH or mild alkaline conditions deacetylates phenolic hydroxyl groups, whereas deacetylation of amino groups by hydroxylamine requires hot acidic or alkaline conditions (Balls & Wood, 1956; Masaik & D'Angelo, 1975). Therefore, we investigated the ability of hydroxylamine to reverse the *N*-acetylimidazole-induced inhibition of the placental $H^+$ pump (Figure 3). In this experiment, cholate-pretreated membrane vesicles were subjected to four different conditions in order to provide proper controls: control vesicles (a); vesicles treated with 2 mM *N*-acetylimidazole (b); vesicles treated with 75 mM hyroxylamine (c); and vesicles treated with 2 mM *N*-acetylimidazole followed by incubation

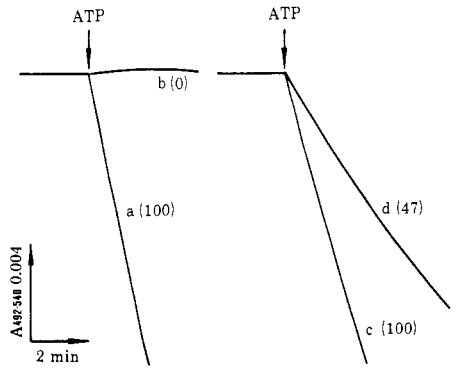


FIGURE 3: Reversal of *N*-acetylimidazole-induced inhibition by hydroxylamine. Cholate-pretreated membrane vesicles were incubated in the presence (curves b and d) or absence (curves a and c) of 2 mM *N*-acetylimidazole for 30 min at room temperature. Following incubation, the vesicles were passed through a Sephadex G-50 column to remove the unreacted reagent. The eluates were divided into two equal parts and incubated with (curves c and d) or without (curves a and b) 75 mM hydroxylamine for 30 min at 37 °C. The vesicles were again passed through a Sephadex G-50 column to remove the unreacted reagent, and the eluates were used for ATP-driven $H^+$-uptake measurements by monitoring the decrease in the absorbance of acridine orange. The values given in parentheses for curves b and d represent $H^+$-pump activity as the percent of activity in their respective controls, curves a and c. The results are from a representative experiment.

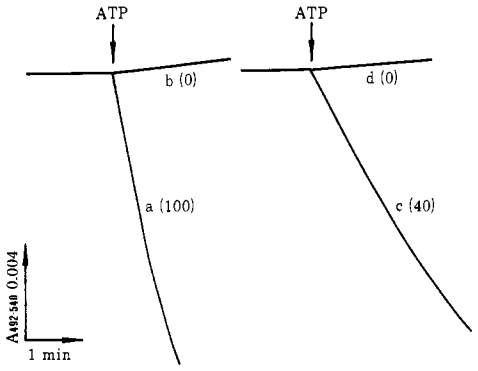


FIGURE 4: Interaction of *N*-acetylimidazole with SH-masked $H^+$ pump. Cholate-pretreated membrane vesicles were incubated in the absence (curve a) or presence (curve b) of 250 $\mu$M pCMBS. The pCMBS-treated vesicles were again incubated with (curve d) or without (curve c) 10 mM *N*-acetylimidazole followed by DTT treatment. In all cases, unreacted reagents were removed after each treatment by filtration through a Sephadex G-50 column. The ATP-driven $H^+$ uptake was measured in these vesicles by monitoring the decrease in the absorbance of acridine orange. The values given in parentheses for curves b–d represent $H^+$-pump activity as the percent of activity in curve a.

with 75 mM hydroxylamine (d). In all cases, unreacted reagents were removed prior to measurement of the $H^+$-pump activity. Treatment of the vesicles with *N*-acetylimidazole caused complete inhibition of the $H^+$ pump (curve b vs curve a), but this inhibition was reversed to a substantial extent by hydroxylamine (curve d vs curve c).

In order to rule out the involvement of thiol groups in the *N*-acetylimidazole-induced inhibition of the human placental $H^+$ pump, we carried out experiments to investigate the interaction of this reagent with the $H^+$ pump whose reactive thiol groups were masked by pCMBS. As shown in Figure 4, treatment of the cholate-pretreated membrane vesicles with 250 $\mu$M pCMBS caused total inactivation of the $H^+$ pump (curve b versus curve a). These SH-masked vesicles, when subsequently treated with or without 10 mM *N*-acetylimidazole, failed to show any detectable $H^+$-pump activity (data not shown). However, the pump activity was recovered

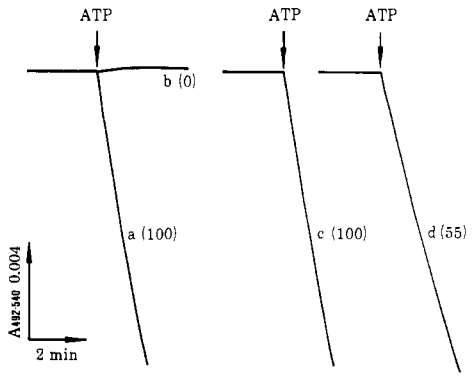


FIGURE 5: Protection of the $H^+$ pump from *N*-acetylimidazole-induced inhibition by ATP. Cholate-pretreated membrane vesicles were incubated with (curves b and d) or without (curves a and c) 3 mM *N*-acetylimidazole in the presence (curves c and d) or absence (curves a and b) of 5 mM ATP. $MgCl_2$ (5 mM) was present in all cases. Following incubation, the vesicles were passed through a Sephadex G-50 column to remove unreacted reagents. The eluted vesicles were used for ATP-driven $H^+$-uptake measurements by monitoring the decrease in the absorbance of acridine orange. The values given in parentheses for curves b and d represent $H^+$-pump activity as percent of activity in their respective controls, curves a and c. The results are from a representative experiment.

to a significant extent upon DTT (25 mM) treatment in SH-masked vesicles which were not exposed to *N*-acetylimidazole (curve c). On the contrary, the SH-masked vesicles which were treated with *N*-acetylimidazole failed to show $H^+$-pump activity upon similar treatment (curve d). If one assumes that pCMBS has modified all the essential thiol groups because it has led to complete inactivation of the $H^+$ pump, there should be no essential thiol groups available in SH-masked vesicles for interaction with *N*-acetylimidazole. Under these conditions, DTT is expected to reactivate the $H^+$ pump in these vesicles if thiol groups are the only potential site of reaction with *N*-acetylimidazole. The fact that DTT failed to reactivate the $H^+$ pump after *N*-acetylimidazole treatment suggests that amino acid residues other than cysteinyl residues are involved in the inhibition of the placental $H^+$ pump by this reagent. However, since the completeness of the reaction of the thiol groups with pCMBS was not monitored, the possibility that the SH-masked vesicles might have contained thiol groups which were inaccessible to pCMBS but reactive toward *N*-acetylimidazole cannot be completely ruled out.

Taken collectively, these data strongly suggest, but do not prove, that the *N*-acetylimidazole-induced inhibition of the placental $H^+$ pump was due to chemical modification of tyrosyl residues. These experiments provide the first supportive evidence for an essential role of tyrosyl residues in the maintenance of optimal activity of a V-type $H^+$ pump from animal cells.

*Protection by ATP from the Inhibition Induced by N-Acetylimidazole.* Protection experiments with ATP have shown that the NEM-reactive thiol groups are located at or near the ATP-binding site of the V-type $H^+$ pumps (Arai et al., 1987; Moriyama & Nelson, 1987; Bowman et al., 1986; Percy & Apps, 1986). We carried out similar experiments to see whether the critical tyrosyl residues, which were found to be essential for the activity of the human placental $H^+$ pump, were located at or near the ATP-binding site. Figure 5 describes the effect of ATP on the N-acetylimidazole-induced inhibition of the placental $H^+$ pump. In this experiment, cholate-pretreated membrane vesicles were subjected to four different experimental conditions: control vesicles (a); vesicles treated with 3 mM *N*-acetylimidazole (b); vesicles treated with 5 mM ATP (c); and vesicles treated with 5 mM ATP plus 3

Table II: Protection of the $H^+$ Pump from *N*-Acetylimidazole-Induced Inhibition by ATP and ADP[a]

| treatment | $H^+$ uptake (%) |
|---|---|
| none | 100 ± 12 |
| *N*-acetylimidazole | 0 |
| *N*-acetylimidazole + ATP | 64 ± 7 |
| *N*-acetylimidazole + ADP | 68 ± 2 |
| *N*-acetylimidazole + adenosine | 0 |
| *N*-acetylimidazole + adenine | 0 |

[a] For details, see Materials and Methods. The final concentration of *N*-acetylimidazole was 3 mM, and that of protecting compounds was 5 mM. The results, given as means ± SE, are from four to six determinations done with two to three different membrane preparations.

Table III: Protection of the $H^+$ Pump by ATP from the Inhibition Induced by NBD-Cl, pNBSF, and Tetranitromethane[a]

| treatment | $H^+$ uptake (%) |
|---|---|
| NBD-Cl | |
| control | 100 |
| inhibitor (10 μM) | 12 |
| inhibitor (10 μM) + ATP–$Mg^{2+}$ (5 mM) | 83 |
| pNBSF | |
| control | 100 |
| inhibitor (1 mM) | 0 |
| inhibitor (1 mM) + ATP–$Mg^{2+}$ (5 mM) | 41 |
| tetranitromethane | |
| control | 100 |
| inhibitor (25 μM) | 27 |
| inhibitor (25 μM) + ATP–$Mg^{2+}$ (5 mM) | 70 |

[a] For details, see Materials and Methods. The results are from a single experiment done in duplicate.

mM *N*-acetylimidazole (d). $MgCl_2$ was present in all cases at a concentration of 5 mM. Comparison of the pump activity in control vesicles (curve a) and in vesicles treated with *N*-acetylimidazole alone (curve b) revealed that *N*-acetylimidazole completely inhibited the $H^+$ pump. However, when the activity of the $H^+$ pump was compared between the vesicles which were treated with ATP (curve c) and the vesicles which were treated with ATP plus *N*-acetylimidazole (curve d), it was obvious that *N*-acetylimidazole was unable to inhibit the $H^+$ pump completely in the presence of ATP. These data show that ATP, a substrate for the $H^+$ pump, offers protection for the pump from the inhibition induced by *N*-acetylimidazole.

*Specificity of Protection.* Table II compares the abilities of ATP and related compounds to protect the placental $H^+$ pump from the *N*-acetylimidazole-induced inhibition. ATP as well as ADP was found to be capable of offering protection whereas adenosine and adenine were not. The protection by ADP was explainable because ADP competes for the ATP-binding site. Adenosine and adenine, on the other hand, do not interact with the site and thus do not possess the ability to protect the pump.

*Protection by ATP from the Inhibition of the $H^+$ Pump Induced by Other Tyrosyl Group Specific Reagents.* We also investigated the ability of ATP to protect the human placental $H^+$ pump from the inhibition induced by three other tyrosyl group specific reagents, namely, NBD-Cl, pNBSF, and tetranitromethane. The results, given in Table III, show that ATP offers considerable protection in all three cases.

The results from the above-described protection experiments strongly suggest that the location of the critical tyrosyl groups and the ATP-binding site interact with each other sterically and/or allosterically. In the case of steric interaction, the presence of ATP would mask the critical tyrosyl groups and thus render them inaccessible to reaction with the modifying reagents. In the case of allosteric interaction, the critical tyrosyl groups may be located at a site spatially distinct from the ATP-binding site, but the presence of ATP would still protect the tyrosyl groups from chemical modification by inducing conformational changes in the $H^+$ pump and thus make the groups unreactive toward the modifying reagents.

Table IV: Influence of Treatment with *N*-Acetylimidazole on the Kinetic Parameters of the Placental $H^+$ Pump[a]

| treatment | $K_m$(ATP) (mM) | $V_{max}$ [$\Delta A$/(mg·min)] |
|---|---|---|
| control | 0.46 ± 0.04 | 0.0674 ± 0.0032 |
| *N*-acetylimidazole | 0.61 ± 0.04 | 0.0465 ± 0.0017 |

[a] Cholate-pretreated membrane vesicles were incubated with or without *N*-acetylimidazole (0.3 mM) at room temperature for 30 min. The vesicles were then passed through a Sephadex G-50 column, and the control and treated eluates were used for measuring the $H^+$-pump activity. ATP concentration varied over the range of 0.2–2.0 mM. Kinetic constants were calculated from Eadie–Hofstee plots (velocity/substrate concentration versus velocity). The plots were linear ($r^2 > 0.97$) for control as well as treated vesicles. The results, given as means ± SE, are from four determinations done with two membrane preparations.

*Effect of N-Acetylimidazole Treatment on the Kinetic Parameters of the Placental $H^+$ Pump.* To determine the influence of the chemical modification of the critical tyrosyl residues on the kinetics of the $H^+$ pump, we calculated the kinetic constants for the pump in control vesicles and in vesicles treated with 0.3 mM *N*-acetylimidazole (Table IV). Treatment with the tyrosyl group specific reagent significantly reduced the affinity of the pump for ATP [apparent dissociation constant for ATP ($K_m$): 0.46 ± 0.04 mM in control vesicles and 0.61 ± 0.04 mM in treated vesicles]. Similarly, the maximal velocity ($V_{max}$) of the pump was also significantly less in *N*-acetylimidazole-treated vesicles than in control vesicles [0.0465 ± 0.0017 vs 0.0674 ± 0.0032 $\Delta A$/(mg·min)].

*Role of Tyrosyl Residues in the Catalytic Activity of Other $H^+$ Pumps.* NBD-Cl is a well-known inhibitor of the mitochondrial $H^+$-ATPase ($F_0$–$F_1$ type). Ferguson et al. (1974, 1975) have shown that the inactivation of the enzyme by NBD-Cl is due to chemical modification of a single tyrosyl residue. This NBD-Cl-reactive residue has subsequently been identified as Tyr-311 of the β-subunit of the $H^+$-ATPase (Andrews et al., 1984; Sutton & Ferguson, 1985). This tyrosyl residue apparently resides within the ATP-binding site of the enzyme because chemical modification with NBD-Cl results in the inability of the enzyme to bind arylazido derivatives of nucleotides (Lunardi & Vignais, 1979). Even though the V-type $H^+$-ATPases ($H^+$ pumps) are also strongly inhibited by micromolar concentrations of NBD-Cl, the identity of the reactive amino acid residue has not been established. The amino acid sequences, either complete or partial, of the catalytic subunit of various V-type $H^+$ pumps are currently known. These include the $H^+$ pumps of the following cellular origins: plant [*Daucus carota* (Zimniak et al., 1988)], fungus [*Neurospora crassa* (Bowman et al., 1988)], archaebacterium [*Sulfolobus acidocaldarius* (Denda et al., 1988)], yeast [*Saccharomyces cerevisiae* (Hirata et al., 1990)], and animal [bovine (Sudhof et al., 1989)]. There is a considerable homology between the amino acid sequences of the catalytic subunits of mitochondrial $H^+$-ATPases and V-type $H^+$ pumps (Zimniak et al., 1988; Bowman et al., 1988; Gogarten et al., 1989). However, there is no tyrosyl residue in the amino acid sequences of the V-type $H^+$ pumps which corresponds to the NBD-Cl-reactive Tyr-311 of the mitochondrial $H^+$-ATPase. Instead, a seryl residue is present in that position in all of the V-type $H^+$ pumps (Ser-407 in *Neurospora crassa*, Ser-414 in *Daucus carota*, Ser-392 in *Sulfolobus acidocaldarius*, and Ser-872 in *Saccharomyces cerevisiae*). However, it is unlikely that the aliphatic hydroxyl group of the seryl residue reacts

with NBD-Cl as does the phenolic hydroxyl group of tyrosyl residues. In fact, Uchida et al. (1988) have provided strong supporting evidence through chemical and kinetic studies that the V-type $H^+$ pump of *Saccharomcyes cerevisiae* is inactivated by NBD-Cl via chemical modification of a single tyrosyl residue. Moreover, we have shown in the present study that, in addition to NBD-Cl, three other reagents which are specific toward tyrosyl residues inactivate the V-type $H^+$ pump of the placental brush-border membrane. Therefore, a more likely explanation is that the reactive tyrosyl residue is located somewhere else in the V-type $H^+$ pumps.

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**Registry No.** ATPase, 9000-83-3; $H^+$, 12408-02-5; ATP, 56-65-5; tyrosine, 60-18-4.

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