Role of membrane-associated thiol groups in the functional regulation of gastric microsomal ($H^+ + K^+$)-transporting ATPase system

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(Received 3 February 1983/Accepted 9 May 1983)

The distribution of free thiol groups associated with the membrane proteins of the purified pig gastric microsomal vesicles was quantified, and the relation of thiol groups to the function of the gastric ($H^+ + K^+$)-transporting ATPase system was investigated. Two different thiol-specific agents, carboxypyridine disulphide (CPDS) and *N*-(1-naphthyl)maleimide (NNM) were used for the study. The structure–function relationship of the membrane thiol groups was studied after modification by the probes under various conditions, relating the inhibition of the ($H^+ + K^+$)-transporting ATPase to the ATP-dependent H^+ accumulation by the gastric microsomal vesicles. On the basis of the extent of stimulation of the microsomal ($H^+ + K^+$)-transporting ATPase in the presence and absence of valinomycin (val) about 85% of the vesicles were found to be intact. CPDS at 1 mM completely inhibits the valinomycin-stimulated ATPase and the associated *p*-nitrophenyl phosphatase with a concomitant inhibition of vesicular H^+ uptake. Both the enzyme and dye-uptake activities were fully protected against CPDS inhibition when the treatment with CPDS was carried out in the presence of ATP. ATP also offered protection (about 65%) against NNM inhibition of the ($H^+ + K^+$)-transporting ATPase system and vesicular H^+ uptake. Under similar conditions ATP also protected about 10 and 6 nmol of thiol groups/mg of protein respectively from CPDS and NNM reaction. Our data suggest that the thiol groups on the outer surface of the vesicles are primarily involved in gastric ($H^+ + K^+$)-transporting ATPase function. Furthermore, at least about 15% of the total microsomal thiol groups appear to be associated with the ATPase system. The data have been discussed in terms of the structure–function relationship of gastric microsomes.

Numerous reports in the literature demonstrate that plasma-membrane-associated thiol groups of various cell types are involved in many transport functions (Klip *et al.*, 1979; Erlichman *et al.*, 1979; Becker & Duhn, 1979). The importance of thiol groups in HCl secretion has been demonstrated using bullfrog gastric mucosa *in vitro*, where the thiol-specific agent *p*-chloromercuribenzenesulphonate, when added to the mucosal solution, reversibly inhibits gastric H^+ transport (Solberg & Forte, 1971; Ray & Tague, 1978).

The biochemical machinery involved in the transport of H^+ has recently been identified and localized at the apical and tubulovesicular membranes of the acid-secreting cells of the fundic mucosa (Sachs *et al.*, 1978; Forte *et al.*, 1980; Ray

Abbreviations used: ($H^+ + K^+$)-ATPase, ($H^+ + K^+$)-transporting ATPase; CPDS, carboxypyridine disulphide; NNM, *N*-(1-naphthyl)maleimide; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

& Fromm, 1981). Purified microsomes, derived primarily from the apical and tubulovesicular membranes of the parietal cells, appear in the form of tightly sealed vesicles and are highly enriched in ($H^+ + K^+$)-ATPase (Forte *et al.*, 1974). The microsomal vesicles are capable of accumulating H^+ in the presence of Mg^{2+} , K^+ and ATP under appropriate conditions (Sachs *et al.*, 1978; Forte *et al.*, 1980; Ray & Fromm, 1981) and the process is mediated by the gastric ($H^+ + K^+$)-ATPase system (Sachs *et al.*, 1976). The bulk of the evidence suggests that nearly all of the K^+ -stimulated ATP-hydrolytic sites are exposed to the vesicle exterior. The gastric microsomal vesicles have recently been demonstrated to be a useful model system to study some aspects of structure–function relationships of the biomembranes (Ray *et al.*, 1980; Sen *et al.*, 1980).

Recent reports from several laboratories (Lee *et al.*, 1979; Forte *et al.*, 1981; Schrijen *et al.*, 1981) demonstrated the importance of thiol groups in the

function of gastric microsomal ($H^+ + K^+$)-ATPase. However, there is no quantitative information regarding the orientation of the membrane-associated thiol groups. Furthermore, the details on the fraction of the microsomal thiol groups involved in the function of gastric ($H^+ + K^+$)-ATPase are also unknown. In view of our reports on the topology and function of membrane-associated amino groups in pig gastric microsomes (Ray *et al.*, 1980; Sen *et al.*, 1980; Sen & Ray, 1981) it appears important to study the orientation and role of the microsomal thiol groups in order to have a better understanding of these gastric membranes. In the present paper we report the effects of two thiol agents, namely CPDS and NNM, to study the orientation and role of membrane-associated thiol groups in gastric ($H^+ + K^+$)-ATPase function. Of the two probes, CPDS appears to be relatively impermeable compared with NNM. Effects of binding of the membrane thiol groups with probes on the microsomal ($H^+ + K^+$)-ATPase activity and vesicular H^+ uptake have been studied under a variety of different conditions. Considering about 85% of the vesicles are intact, it appears that about 15% of the microsomal thiol groups are exposed to the exterior, 18% are in the interior and the rest (about 67%) are present in such states that none of the probes can interact with them. The microsomal thiol groups exposed primarily to the vesicle exterior appear to be critically involved in the function of the gastric ($H^+ + K^+$)-ATPase system.

A preliminary report of part of this work has been published (Nandi *et al.*, 1982).

Experimental procedure

Methods

Isolation of gastric microsomes. Fresh pig stomachs were purchased from the local slaughterhouse. The gastric microsomal membranes were harvested by a procedure described previously (Ray, 1978).

Assay of ATPase and *p*-nitrophenyl phosphatase. The ATPase was assayed as previously described (Ray, 1978). Briefly, the incubation mixture contained, in a total volume of 1 ml, 50 μ mol of Pipes (1.4-piperazinediethanesulphonic acid) buffer (pH 6.8), 1 μ mol of $MgCl_2$, 2 μ mol of Tris/ATP, 20 μ g of membrane protein with or without 150 μ mol of KCl in the presence and in the absence of 5 μ M-valinomycin. After 10 min incubation at 21°C the reactions were stopped by 1 ml of 12% trichloroacetic acid. P_i was assayed by the procedure of Sanui (1974).

For *p*-nitrophenyl phosphatase the incubation mixture contained, in a total volume of 1 ml, 50 μ mol of Pipes buffer (pH 6.8), 2 μ mol of $MgCl_2$, 5 μ mol of *p*-nitrophenyl phosphate, 10 μ g of membrane protein, with and without 20 μ mol of KCl. After 20 min

of incubation at room temperature the reactions were stopped by 1 ml of 1.5 M-NaOH. After a brief centrifugation, the absorbance of the supernatant was read at 410 nm.

Study of vesicular H^+ uptake. Vesicular accumulation of H^+ was measured at room temperature by the method of Lee & Forte (1978) and as described previously (Ray *et al.*, 1980; Sen *et al.*, 1980). The amount of Acridine Orange taken up is a sensitive measure of intravesicular H^+ concentration. Wavelengths used were 493–530 nm (excitation→emission) in the Aminco Bowman spectrofluorimeter.

Estimation of thiol groups. The gastric microsomal thiol groups were quantified using two different methods; the DTNB method (Ellman, 1959) and the CPDS method (Mehrisi & Grassetti, 1969).

DTNB method. Gastric microsomes (0.5 mg) were taken up in 1.8 ml of a medium consisting of 10 mM-phosphate buffer (pH 8.0) and 1% sodium dodecyl sulphate. To this, 0.2 ml of 10 mM-DTNB prepared in 10 mM-phosphate buffer (pH 7.0) was added and the resulting suspension was mixed thoroughly. A blank without any microsomes was run in parallel. The mixtures were kept at room temperature for 20 min and the absorbance was read at 412 nm in a Beckman DU spectrophotometer. An ϵ of 13600 $M^{-1} \cdot cm^{-1}$ was used to calculate the concentration of reacting thiol groups (Ellman, 1959).

CPDS method. The reaction between thiol groups and impermeant (Mehrisi & Grassetti, 1969) CPDS (peak of u.v. absorption at 290 nm) leads to the formation of mixed disulphide bonds (Mehrisi & Grassetti, 1969). The disulphide formed between cell thiol groups and CPDS can be split by treatment with thiol reagents such as cysteine, reduced glutathione or dithiothreitol, with the resultant formation of thiones (absorption peak, 344 nm), which are released into the supernatant. Quantification of the released thiones give an estimate of the number of thiol groups exposed on the membrane surface.

Gastric microsomes (0.5 mg) were treated with 0.5–1 mM-CPDS in 250 mM-sucrose/0.5 mM-EDTA/Pipes buffer (pH 7.0) for 5–15 min at room temperature and washed four times with the buffer to remove the excess reagent and the thione released in the primary reaction. The treated microsomes were then allowed to react with reduced glutathione (1 mM) to release the thione bound to the vesicle exterior into the supernatant, which was then measured spectrophotometrically. Details for any particular experiment are given in appropriate places in the Figure and Table legends.

Materials

Pipes, Tris, AMP, ADP, ATP, CTP, GTP, ITP, *p*-nitrophenyl phosphate, CPDS, DTNB and valino-

mycin were purchased from Sigma Chemical Company. Acridine Orange was purchased from Aldrich and dimethyl sulphoxide from Fisher Chemical Company. NNM was purchased from Molecular Probes, Plano, TX, U.S.A. All other chemicals used were the best grades available on the market.

Results

Effects of CPDS and NNM on the microsomal ($H^+ + K^+$)-ATPase and K^+ -dependent p -nitrophenyl phosphatase activities

Comparison of the K^+ -stimulated rate of ATP hydrolysis at zero probe in the presence and in the absence of valinomycin demonstrates that valinomycin stimulates nearly 5-fold compared with that with K^+ alone (Fig. 1). These data suggest that about 85% of the vesicles are impermeable to K^+ , and hence need valinomycin to allow K^+ to enter into the vesicle interior for the subsequent stimulation of ATPase activity. Effects of treatment of gastric microsomes with different concentrations of CPDS on gastric microsomal ATPase activity are shown in Fig. 1(a). Whereas the ATPase activity in presence of Mg^{2+} plus K^+ (K^+ -stimulated) remained unaltered, the activity in presence of K^+ and valinomycin (valinomycin-stimulated) decreased linearly with increasing CPDS concentration. The valinomycin-stimulated activity was abolished at 1 mM-CPDS. The data in Fig. 1(b) show that the inhibition of valinomycin-stimulated activity by 1 mM-CPDS is biphasic. There was a rapid inhibition within 2.5 min, followed by slow and almost linear inhibition, the activity being abolished after 10–15 min of CPDS treatment (Fig. 1b).

The data on the effects of different concentrations of NNM (Fig. 4a) on the gastric ATPase activity show that, like CPDS (Fig. 1a), the basal and K^+ -stimulated activities are not appreciably altered. However, the valinomycin-stimulated activity decreases rapidly between 0.25 mM- and 0.5 mM-NNM, the activity being abolished at the latter concentration. It is noteworthy that unlike CPDS (Fig. 1a) the valinomycin-stimulated activity was reduced at a very low rate below 0.25 mM-NNM (Fig. 2a). Effects of time of pre-incubation of gastric microsomes with 0.5 mM-NNM demonstrated that the valinomycin-stimulated activity was abolished within 15 min (Fig. 2b).

Protective effects of ATP on the inactivation of valinomycin-stimulated ATPase and p -nitrophenyl phosphatase activities by CPDS are shown in Table 1. The presence of ATP (0.1 mM or less) during CPDS treatment almost completely protects the enzyme from CPDS inactivation. Similar protective effects of ATP on the inactivation of microsomal K^+ -stimulated p -nitrophenyl phosphatase activity by CPDS is observed (Table 1). In contrast with CPDS, the NNM inhibition of the enzyme activities could be protected to the extent of about 60% in the presence of ATP. Similar protection (Table 1) was observed if the microsomes were pre-exposed to ATP and subsequently treated with CPDS or NNM alone (J. Nandi, Z. Meng-Ai & T. K. Ray, unpublished work). Thus, the requirement for ATP in the protection of the gastric ATPase system for CPDS or NNM inhibition are indeed very small. The effects of different nucleotides on the protection of valinomycin-stimulated ATPase against thiol-reac-

Fig. 1. Effects of increasing concentrations of CPDS on gastric microsomal ($H^+ + K^+$)-ATPase activity (a) and effects of CPDS treatment of gastric microsomes for different lengths of time on the microsomal ATPase activity (b)

(a) The microsomes were treated with different concentrations of CPDS for 15 min at 0–4°C as described in the legend to Table 2. Portions of the control and CPDS-treated microsomes were assayed for the ATPase activity as described under 'Methods'. Symbols: ○, Mg^{2+} alone; ●, Mg^{2+} plus 150 mM- K^+ ; △, Mg^{2+} plus K^+ plus 5 μM-valinomycin. Data are means ± S.E.M. ($n = 4$). (b) Microsomes were treated with 1 mM-CPDS under similar conditions as for (a) for different times. Portions were assayed for ATPase activity. Details of the ATPase assay are given under 'Methods'. Symbols: ○, Mg^{2+} alone; ●, Mg^{2+} plus 150 mM- K^+ ; △, Mg^{2+} plus K^+ plus 5 μM-valinomycin. Data are means ± S.E.M. ($n = 4$).

Fig. 2. Effects of increasing concentrations of NNM on gastric microsomal ($H^+ + K^+$)-ATPase activity (a) and effects of treatment of gastric microsomes for different time periods with NNM on gastric ($H^+ + K^+$)-ATPase activity (b)

(a) Microsomes were treated with different concentrations of NNM (0–1 mM) in 250 mM-sucrose/2 mM-Pipes/0.5 mM-EDTA (pH 6.8) at 0–4°C. NNM was dissolved in 25% dimethyl sulphoxide and the final concentration of dimethyl sulphoxide in the membrane incubation system was 2.5%. Control microsomes were treated with 2.5% dimethyl sulphoxide alone. After 15 min the membranes were diluted 20-fold with ice-cold Pipes buffer (pH 6.8) as above and processed by the procedure described in the legend to Table 2. Portions of the microsomes were assayed for the ATPase activity in the presence of Mg^{2+} alone (○), Mg^{2+} plus 150 mM- K^+ (●) and Mg^{2+} plus K^+ plus 5 μM-valinomycin (▲). Details of the ATPase assay are given under 'Methods'. (b) Membranes were treated with 0.5 mM-NNM. Details are given in (a). Symbols: ○, Mg^{2+} alone; ●, Mg^{2+} plus 150 mM- K^+ ; ▲, Mg^{2+} plus K^+ plus 5 μM-valinomycin. Data are means $\pm$ S.E.M. ($n = 4$).

Table 1. Protection of microsomal ($H^+ + K^+$)-ATPase and *p*-nitrophenyl phosphatase activities against CPDS or NNM inactivation by ATP

Microsomes were treated with either 1 mM-CPDS or 0.5 mM-NNM in the presence and in the absence of 0.1 mM-ATP at 0–4°C for 15 min. See the legend to Fig. 1 for the details of CPDS treatment and the legend to Fig. 2 for the details of NNM treatment. Control membranes were run in parallel under identical conditions without any probe. At the end of 15 min the microsomes were diluted 20-fold with ice-cold 250 mM-sucrose/2 mM-Pipes/0.5 mM-EDTA (pH 6.8) and harvested by centrifugation at 100 000 *g* for 90 min at 0–4°C. The pellets were resuspended in ice-cold 250 mM-sucrose/2 mM-Pipes/0.5 mM-EDTA (pH 6.8) and portions were tested for ATPase and *p*-nitrophenyl phosphatase activities at room temperature. Details of the assay are given under 'Methods'. The basal (with Mg^{2+} as the only cation) activities were not affected by CPDS or NNM to any appreciable extent and the activities (μmol/mg of protein per h) of the ATPase in groups A and B were 1.2 and 4.0 and of *p*-nitrophenyl phosphatase were 0.28 and 0.16 respectively. Data are means $\pm$ S.E.M. ($n = 4$).

Treatment	Enzyme activity (μmol/mg of protein per h)			K ⁺ -stimulated <i>p</i> -nitrophenyl phosphatase
	K ⁺ -stimulated ATPase			
	Without valinomycin (i)	With valinomycin (ii)	(ii) – (i)	
(A) None (control)	3.23 ± 0.13	11.38 ± 0.13	8.15	5.94 ± 0.31
CPDS (1 mM)	2.6 ± 0.23	2.56 ± 0.80	0	1.96 ± 0.02
CPDS + ATP	1.8 ± 0.63	9.84 ± 0.3	8.0	5.91 ± 0.06
(B) None (control)	1.0 ± 0.4	5.3 ± 0.2	4.3	6.1 ± 0.3
NNM (0.5 mM)	0.95 ± 0.4	1.0 ± 0.6	0.05	2.06 ± 0.14
NNM + ATP	0.8 ± 0.35	2.95 ± 0.25	2.15	3.9 ± 0.08

tive agents showed that GTP was equally effective as ATP, whereas other nucleotides such as CTP, ITP, ADP and AMP were without any effect (J. Nandi, Z. Meng-Ai & T. K. Ray, unpublished work). It should be pointed out that abolition of the valinomycin-stimulated ATPase activity by CPDS and NNM is not due to an inactivation of valinomycin by those agents since the microsomes were nearly freed from the unreacted thiol agents by

20-fold dilution and centrifugation before testing the valinomycin effect (Table 1 and Figs. 1 and 2). This is also consistent with the ATP protection of the valinomycin-stimulated ATPase against inactivation by the thiol-specific agents (Table 1).

In separate studies we tested the effects of pretreatment of microsomal vesicles by CPDS or NNM on the stimulation of gastric ATPase activity in the presence of K^+ and gramicidin. Similar to their

effects on valinomycin-stimulated activity, the probes completely inhibited the gramicidin-stimulated ATPase activity (results not shown). Furthermore, the K^+ -stimulated activity associated with the leaky membranes obtained by repeated freezing and thawing in hypo-osmotic medium was also totally abolished by pre-exposure of microsomes to 2 mM-CPDS or to 1 mM-NNM at room temperature within 30 min (J. Nandi, Z. Meng-Ai and T. K. Ray, unpublished work). It may be noted that, unlike the intact vesicles, the leaky microsomes become totally insensitive to valinomycin with respect to optimal K^+ -stimulated ATPase activity. The study also revealed that even though both the K^+ -stimulated activity of the leaky membranes and the valinomycin-stimulated activity of the intact vesicles are inhibited by CPDS and NNM, the former enzyme activity was relatively resistant and required a more drastic treatment than the latter for maximal inhibition. These observations suggested that the valinomycin-independent part of the K^+ -stimulated ATPase activity of the intact vesicles that was not inhibited by the probes under the conditions of our study (Figs. 1 and 2) is due partially to some contaminating leaky membranes. However, the reasons for the differential sensitivity of the K^+ -stimulated ATPase activity associated with the intact and leaky vesicles to the thiol-specific agents remains unclear at present.

Effects of treatment of gastric microsomes with CPDS or NNM on ($H^+ + K^+$)-ATPase-mediated vesicular H^+ uptake

Effects of pretreatment of gastric microsomes with different concentrations of CPDS on valinomycin-induced Acridine Orange uptake by the gastric microsomal vesicles were studied. Fig. 3(a) shows that the dye-uptake ability of the microsomes was greatly reduced under conditions when the valinomycin-stimulated activity was completely inhibited (Table 1). Similar to the valinomycin-stimulated ATPase activity (Table 1), the dye-uptake activity was also not appreciably affected when the microsomes were treated with CPDS in the presence of ATP (Fig. 3b).

A good correlation between the effect of valinomycin-stimulated ATPase and vesicular Acridine Orange uptake was also observed with NNM. Thus, up to 0.3 mM, the inhibitory effects of NNM on ATPase (Fig. 2a) and dye uptake (Fig. 4a) were at a very low level. However, at 0.5 mM or higher, both the valinomycin-stimulated ATPase activity (Fig. 2a) and dye-uptake activity (Fig. 4a) were almost completely inhibited. The presence of ATP during NNM treatment protected the ATPase (Table 1) and Acridine Orange uptake (Fig. 4b) to nearly the same extent.

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Fig. 3. Gastric ($H^+ + K^+$)-ATPase-mediated Acridine Orange uptake by microsomal vesicles pretreated with different concentrations of CPDS (a) and protective effects of ATP against CPDS inhibition of gastric ($H^+ + K^+$)-ATPase-mediated vesicular H^+ uptake (b)

(a) Conditions for CPDS treatment of gastric microsomes were the same as described in the legend to Fig. 1. Various concentrations (mM) of CPDS used are indicated on the graph. The incubation system contained, in a total volume of 2 ml, 25 mM-Pipes buffer (pH 7.0) containing 0.5 mM-EDTA, 1 mM- Mg^{2+} , 150 mM- K^+ , 0.01 mM-Acridine Orange and 0.01 mg of membrane protein. The reactions were initiated at room temperature with 5 μ M-valinomycin and the fluorescence quenching of Acridine Orange was monitored as described under 'Methods'. (b) Conditions for the CPDS (1 mM) treatment of gastric microsomes in the presence and in the absence of ATP were the same as in Table 1. Vesicular dye uptake measurement and conditions were as in (a).

Interaction of CPDS and NNM with thiol groups associated with gastric microsomal vesicles

The data in Table 2 show that the total microsomal thiol groups obtained by the CPDS (Mehrisi & Grassetti, 1969) and DTNB (Ellman, 1959) methods in the presence of sodium dodecyl sulphate are nearly identical. These values (Table 2) for total microsomal thiol groups are almost identical with those (80 nmol/mg) recently reported (Lee *et al.*, 1979) using the DTNB method.

Fig. 5 shows the effects of different concentrations of CPDS or NNM on the labelling of microsomal thiol groups when reacted for 15 min. As the concentrations were raised from 0.25 to 1.0 mM the labelling by both CPDS and NNM increased significantly. About 0.5 mM was found to

Fig. 4. Effects of pretreatment of gastric microsomes with different concentrations of NNM on $(H^+ + K^+)$ -ATPase-mediated vesicular H^+ uptake (a) and protective effects of ATP against NNM inhibition of vesicular H^+ uptake (b).

(a) Details of NNM treatment are given in the legend to Fig. 2(a). Conditions of Acridine Orange uptake are given in the legend to Fig. 3(a). The control system contained 2.5% dimethyl sulphoxide (DMSO). (b) Conditions of treatment of gastric microsomes with 0.5 mM-NNM in the presence and in the absence of ATP are described in Table 1. Details of the dye uptake study are given in the legend to Fig. 3(a) and under 'Methods'.

Fig. 5. Binding of microsomal free thiol groups by different concentrations of CPDS or NNM.

The microsomes (0.5–1 mg) were treated with CPDS (○) or NNM (●) at 0–4°C for 15 min. Details are given in the legend to Table 2 and under 'Methods'. Data are means $\pm$ S.D. ($n = 6$).

be the optimal concentration for both the agents (Fig. 5).

The kinetics of binding of the reactive thiol groups of gastric microsomes with CPDS (1 mM) or NNM

Table 2. Quantification of microsomal thiol groups reactive to CPDS or NNM under various conditions. Treatment with CPDS or NNM was conducted at 0–4°C for the indicated time. The microsomes were pre-incubated without (control) or with 1 mM-CPDS or 0.5 mM-NNM in 250 mM-sucrose/2 mM-Pipes/0.5 mM-EDTA (pH 6.8). At the end of incubation the reaction mixture was diluted 20-fold with ice-cold buffer as described above and harvested by centrifugation at 100000g for 90 min. The pellets after resuspension in the same buffer were used for the estimation of unchanged thiol groups. The unchanged thiol groups were quantified by the DTNB method after solubilization with 1% sodium dodecyl sulphate. The number of unreacted groups were calculated by subtracting the values for unchanged thiol groups from the total microsomal thiol groups. Details for the thiol-group assay are given under 'Methods'. Data are means $\pm$ S.D. n values are given in parentheses. The leaky vesicles were obtained by repeated (three times) and quick freezing and thawing of the membranes in a hypo-osmotic 2 mM-Pipes buffer (pH 6.8) containing 0.5 mM-EDTA. The leaky vesicles thus obtained were used immediately.

Methods used	Thiol groups reacting (nmol/mg of microsomal protein)
1% sodium dodecyl sulphate solubilized (DTNB method)	80.7 $\pm$ 2.4 (14)
1% sodium dodecyl sulphate solubilized (CPDS method)	79.8 $\pm$ 1.4 (8)
DTNB method	
Intact vesicles, 1 mM-CPDS (15 min)	14.4 $\pm$ 1.5 (8)
Leaky vesicles, 1 mM-CPDS (15 min)	26.0 $\pm$ 1.0 (4)
Intact vesicles, 0.5 mM-NNM (15 min)	22.3 $\pm$ 1.0 (8)
Leaky vesicles, 0.5 mM-NNM (15 min)	28.0 $\pm$ 1.4 (4)
Intact vesicles, 0.5 mM-NNM (30 min)	27.0 $\pm$ 1.0 (6)
Intact vesicles, treated sequentially with CPDS and NNM (15 min each)	28.0 $\pm$ 0.5 (4)

(0.5 mM) at 0–4°C are shown in Fig. 6. The report that CPDS is largely a membrane-impermeable agent (Mehrisi & Grassetti, 1969) is consistent with our data (Fig. 6), where the probe, after an initial titration of about 14 nmol within 5 min, shows a slow linear increase in labelling with a 25% elevation at 60 min compared with those at 5 min. The uncharged NNM shows a much higher level of labelling than CPDS throughout the 60 min period (Fig. 6). The labelling with NNM increases rapidly within 5 min and moderately between 5 and 30 min, but changes very slowly thereafter (Fig. 6). Such time-dependent rapid labelling of microsomal thiol groups by NNM strongly suggests that NNM is relatively more permeant into microsomal vesicles than is CPDS.

The protection of microsomal thiol groups against

Fig. 6. Quantification of microsomal thiol groups reactive with different thiol-specific agents as a function of time exposure

Microsomes were treated at $0-4^{\circ}\text{C}$ with 1 mM-CPDS (O) or 0.5 mM-NNM (●) in 250 mM-sucrose/0.5 mM-EDTA/2 mM-Pipes buffer (pH 6.8) for the designated time as described in Table 2. The membrane-associated thiol groups remaining after conjugation with the probes were quantified by the DTNB procedure (see under 'Methods'). The amount of probe bound with microsomal thiol groups at any given time was calculated by subtracting the value obtained after probe conjugation from the total thiol groups in untreated control microsomes.

interaction with CPDS and NNM in the presence of ATP is shown in Table 3. ATP protects about 10 and 6 nmol of membrane thiol groups against CPDS and NNM conjugation respectively (Table 3). Such protection of membrane thiol groups correlates well with the protection of gastric ($H^+ + K^+$)-ATPase activity (Table 1) and vesicular H^+ uptake ability of the microsomes (Figs. 3 and 4) by ATP against inhibition by the thiol-specific agents. These observations are suggestive of some ATP-induced conformational changes in the gastric ATPase system and are consistent with the reports of Schrijen *et al.* (1980) and Lee & Forte (1980). Whether the suggested ATP-induced conformational change is due to binding of the nucleotide itself or to phosphorylation of the enzyme protein remains to be elucidated.

Discussion

Distribution of thiol groups across gastric microsomal vesicles

The data in Fig. 6 demonstrate that after a very rapid interaction within 5 min the labelling of microsomal thiol groups by 1.0 mM-CPDS increases at a much lower rate than with 0.5 mM-NNM. Also, NNM shows significantly higher levels of labelling than CPDS at all time points. Our data (Fig. 6) are consistent with the reports that the negatively charged probe CPDS (Mehrisi & Grassetti, 1969) is

Table 3. Protection of microsomal thiol groups against interaction with CPDS and NNM in the presence of ATP

Treatment with different thiol-specific agents was carried out at $0-4^{\circ}\text{C}$ for 15 min. Membranes were treated with or without 0.1 mM-ATP. Details for the treatment and assay procedure are given in Table 2 and under 'Methods'. Data are means $\pm$ s.d. ($n = 8$).

Agents used	Microsomal thiol groups (nmol/mg of protein)		
	Without ATP	With ATP	Protected by ATP
CPDS (1 mM)	11.8 ± 1.6	3.8 ± 0.9	10.0
NNM (0.5 mM)	22.1 ± 1.0	16.1 ± 3.4	6.0

relatively impermeant to biomembranes. However, the uncharged NNM, a naphthyl derivative of maleimide, appears to be sufficiently hydrophobic to penetrate biomembranes and this is suggested by our data (Fig. 6).

Of the total (80.7 nmol/mg of protein) microsomal thiol groups (Table 2) only 14.4 and 26 nmol were conjugated by CPDS in the intact and leaky vesicles respectively. In contrast with CPDS, about 22 and 28 nmol were attacked by NNM in the intact and leaky vesicles respectively under similar conditions (Table 2). However, when the intact vesicles were treated with NNM for 30 min (Table 2) instead of for 15 min as above, 27 nmol of the microsomal thiol groups were attacked. Since the total number of thiol groups reactive within 15 min with CPDS and NNM in leaky vesicles was nearly the same as those of the intact vesicles treated with NNM for 30 min, it is highly likely that predominantly the same groups are titrated by either CPDS or NNM under these conditions (Table 2). Such a conclusion would be consistent with the observed absence of change in titratable thiol groups obtained by sequential treatment of the intact vesicles with the two probes (Table 2).

If we consider that about 85% of the vesicles are intact (Sen & Ray, 1981; see the Results section) the number of thiol groups reacting rapidly with the poorly permeant CPDS will be about 10 nmol. These fast-reacting groups would primarily be exposed to the vesicle exterior. The fate of the remaining 12 nmol of reactive thiol groups is not known, although they are likely to be largely located at the vesicle interior, since they were available to CPDS only in the leaky vesicles. The state of most (about 46 nmol) of the microsomal thiol groups, however, remains completely unclear at present.

The thiol groups exposed at the vesicle exterior are critically involved in the function of gastric K^+ -stimulated ATPase

Microsomal valinomycin-stimulated ATPase activity is completely abolished when microsomes are

incubated at 0–4°C with either 1 mM-CPDS (Fig. 1) or 0.5 mM-NNM (Fig. 2) for 15 min. Under the same conditions the thiol groups that react with CPDS and NNM (Fig. 6) amount to about 13.8 and 22 nmol respectively. Inhibitory effects of the thiol-specific agents on valinomycin-stimulated ATPase activity appear to be due to the blockage of some critical thiol groups associated with the gastric K⁺-stimulated ATPase system and not to an inhibition of some Cl⁻ conductance mechanism of the vesicles. The reasons are as follows. Another K⁺-ionophore, gramicidin, whose effect is not restricted by anion conductance, also stimulates the gastric ATPase and the activity could be blocked by both thiol-specific agents under conditions similar to those using valinomycin (see the Results section). Furthermore, the ionophore-independent K⁺-stimulated ATPase activity associated with leaky vesicles obtained by repeated freezing and thawing of the microsomes was also inhibited almost completely by the probes (see the Results section).

The presence of ATP during treatment of the microsomal vesicles with CPDS or NNM protects the enzyme from inactivation. Thus ATP protects the enzyme almost completely from CPDS and by about 65% from NNM inactivation (Table 1). Under identical conditions (15 min exposure at 0–4°C) of enzyme protection ATP also protects a substantial portion of the reactive thiol groups from CPDS and NNM attack (Table 3). Complete protection of the valinomycin-stimulated ATPase (Table 1) and vesicular H⁺ uptake (Fig. 3) by ATP from CPDS inactivation and the simultaneous unavailability of 10 nmol of thiol groups exposed at the vesicle exterior from CPDS attack (Table 3) suggests that those thiol groups protected by ATP are critically involved in gastric ATPase function. It is interesting that ATP protects from NNM attack only 6 nmol or about 60% of the involved thiol groups (Table 3) and also simultaneously protects the valinomycin-stimulated ATPase activity (Table 1) and dye-uptake ability (Fig. 4) of the microsomes to about the same extent. The data (Tables 1 and 3 and Fig. 4) on the ATP protection of NNM effects suggest that either 40% of the total ATPase molecules may remain functionally inactive or the overall efficacies of all the ATPase molecules may be reduced by 40% under such conditions. Although the latter possibility appears to be a more likely one, the former cannot be ruled out altogether.

The present study re-emphasizes the complexity of the structure–function relationship of biomembranes. It is imperative that for a better understanding of the functional aspects of any membrane protein like gastric microsomal (H⁺ + K⁺)-ATPase, many factors, such as details of the tertiary structure, effects of lipid–protein and protein–protein interaction, extent of embedding, exposure and

orientation of various functional groups (including thiol) etc., need not be known.

We thank Miss Susan Golden and Mrs. Lisa Lashway for typing the manuscript. The work was supported in part from the Surgical Research Fund, Upstate Medical Center, and by a grant from U.S. Public Health Service (AM 25544). T. K. R. is the recipient of Research Career Development Award AM 00623.

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