ENERGIZED GASTRIC MICROSOMAL MEMBRANE VESICLES-- AN INDEX USING METACHROMATIC DYES

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SUMMARY Energization of gastric microsomal membrane vesicles by ATP and pH gradient induced dramatic spectral changes on metachromatic dyes such as acridine orange and neutral red. The characteristics of these metachromatic effects are very similar to the known ion transport properties of these vesicles and may prove to be another parameter for monitoring the energization and ion transport processes in these vesicles.

INTRODUCTION In earlier reports from this laboratory, the occurrence of a K^+-stimulated ATPase system has been demonstrated in microsomal preparations from gastric mucosa of frog and pig (1, 2). Since then, evidence has been presented by others to indicate that this microsomal vesicular preparation is able to generate a proton gradient on energization by ATP (3, 4). In this report, we will present evidence that upon energization, these membrane vesicles behave like chromotropic substrates and are able to induce spectral changes in some metachromatic dyes like acridine orange and neutral red. This metachromatic effect may prove to be another parameter for monitoring the energization and ion transport process in these gastric microsomal vesicles.

METHODS A microsomal fraction from pig gastric mucosa was prepared essentially according to the procedure published previously (1, 2) except that the homogenization medium in the present case was 113 mM Mannitol, 37 mM sucrose, 0.2 mM EDTA and 5 mM PIPES buffer (pH 6.68). Both

Abbreviations: PIPES, piperazine-N, N'-bis (2-ethanesulfonic acid); CCCP, carbonyl cyanide, m-chlorophenyl hydrazone; EDTA, ethylenediaminetetra acetic acid; Tris, Tris-(hydroxymethyl)-aminomethane.

the crude microsomes (pellet from 100,000 xg x 90 min centrifugation) and a sucrose density gradient purified fraction (low density microsomal fraction referred to as L1) were used for our investigations. Both fractions showed the same qualitative effect in regard to the spectral shift, but the L1 membranes were quantitatively more active, and since they have been shown to be virtually free of mitochondrial contamination (2) we will report the results on the purified fraction. Absorbance of the dyes was followed with an Aminco-Bowman dual wave length spectrophotometer. The spectra were recorded using a Cary-14 spectrophotometer. In the pH-gradient-induced spectral shift experiments, membrane vesicles together with the dye were incubated in 10 mM succinate at various pH (acid stage) for 1 minute. Then 25 mM Tris base was added to bring the reaction medium to a final pH 8 (base stage). The absorbance was followed continuously with the dual wave length spectrophotometer. All spectral analyses were carried out at room temperature (21-23° C). Acridine orange and neutral red were recrystalized as described by Pal and Schubert (7). All reagents used in this study were of the purest grade available commercially.

RESULTS ATP-induced spectral shifts. Addition of ATP to membrane vesicles incubated in 2 mM $MgCl_2$, 150 mM KCl, 10 μM acridine orange at pH 6.1 induced a large decrease in absorbance at 493 nm. CCCP and valinomycin together, or nigericin alone, restored the initial absorbancy. Figure 1 shows the time course of the O.D. change. The inset of Fig. 1 shows the time course of the alkalization of the medium with addition of ATP. Fig. 2 shows the spectral change induced by ATP and the reversal of the spectrum back to the original one upon addition of ionophores. After the reversal, further addition of ATP cannot induce the spectral shift again. This spectral shift is very similar to the metachromatic effect due to the interaction of the acridine orange with various chromotropic substrates (polyanions) (8). Acridine orange has a pK_a of 10.5; therefore, it is posi-

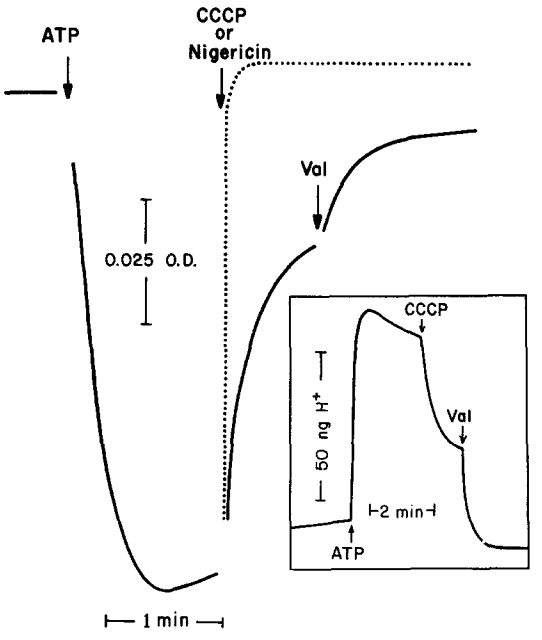

Fig. 1. Time course of ATP-induced spectral changes. Membrane vesicles (0.1 mg protein/ml) were pre-incubated in a medium containing 150 mM KCl, 2 mM $MgCl_2$, 10 mM PIPES (pH 6.1) and 0.2 mM EDTA for 2 hr at room temperature (K^+-Mg^{2+}-medium). 10 μM acridine orange was added and the O. D. changes were recorded using 493 nm as sample wave length and 550 nm as reference. At the time indicated by the arrow, 0.2 mM ATP was added. The solid line shows the sequential effects of CCCP and valinomycin. The dotted line shows the effect of nigericin. All ionophores were added as small aliquots (2-4 μl) in methanol to give final concentration of 2×10^{-5} M. The insert is an analogous experiment in which the pH was recorded. Conditions were the same as above except that membrane protein concentration was 1 mg/ml and no PIPES buffer was included. Addition of 0.8 mM ATP caused an alkalinization of the medium which returned to near base-line levels after successive additions of CCCP and valinomycin (or nigericin, not shown).

tively charged around neutral pH. Upon binding of the positively charged dye molecules to the negative sites along a polyanion chain, the dye molecules can interact with each other (stacking phenomena) and produce a spectral shift, which is the characteristic spectrum of the dimer and/or higher aggregates of the dye. The same metachromatic spectral shift of the dye has been reported on submitochondrial particles upon energization with ATP or succinate plus oligomycin (9, 10, 11).

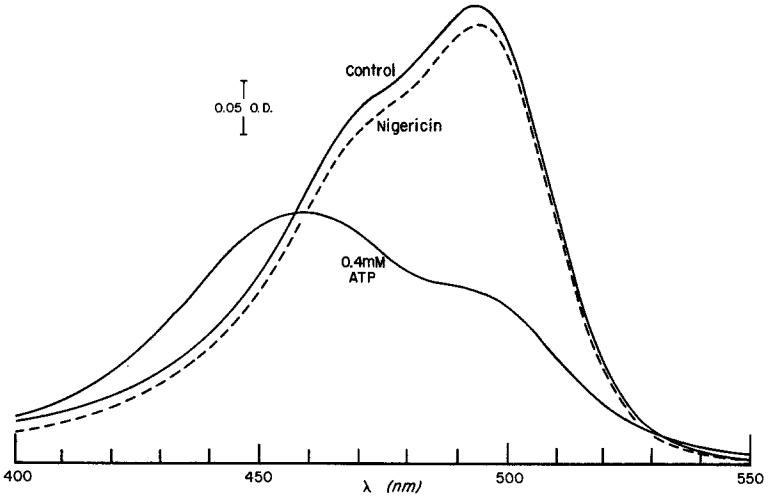

Fig. 2. ATP-induced spectral changes. Conditions same as Fig. I, except that 0.2 mg membrane protein/ml was used and the pre-incubation time was 48 hr at 0-4° C. The control spectrum was 10 μM acridine orange together with membrane vesicles in the K^+-Mg^{2+}-medium at pH 6.1. The shifted spectrum was induced by addition of 0.4 mM ATP. The dotted spectrum shows the reversal produced by adding 2 x 10^{-5} M nigericin to the ATP shifted spectrum.

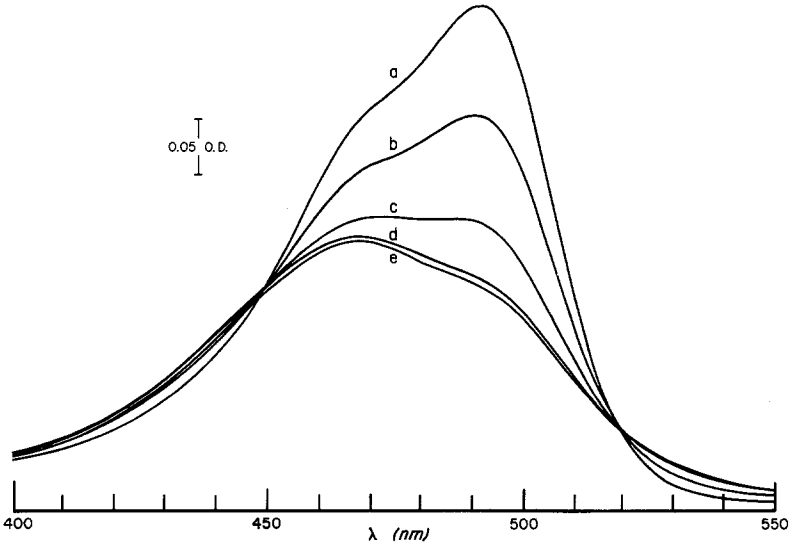

Fig. 3. Spectral changes induced by addition of polystyrene sulfonic acid (PSA). (a) 10 μM acridine orange in K^+-Mg^{2+}-medium (pH 6.1); total volume 1 ml. (b), (c), (d) and (e) show the spectral changes induced by sequential addition of 5 μl aliquots of PSA (425 μg/ml) to (a).

Fig. 3 shows the metachromatic effect of acridine orange induced by addition of polystyrene sulfonic acid (polyanion). Further support for the interpretation that the spectral shift is the result of metachromatic interaction between the dye molecules and the membrane is that another metachromatic dye, neutral red, shows the same behavior when it interacts with the energized membrane vesicles.

The metachromatic effect has specificity for the nucleotide used. ITP, GTP and AMP are ineffective. ADP is partially effective, while p-nitrophenylphosphate is ineffective.

The metachromatic effect induced by energization with ATP required the presence of both Mg^{2+} and K^+. In the case of K^+, preincubation in 150 mM KCl either in cold overnight or 2 hrs at room temperature produced the maximum effect. This suggests that K^+ is required inside the vesicles to produce the maximum effect.

Either $ZnSO_4$ (2 mM) or NaF (2 mM) inhibit both the metachromatic effect and the ATPase. Whereas, oligomycin is ineffective up to 4 μg/mg protein in inhibiting the metachromatic effect indicating that the effect is not due to mitochondrial contamination.

The metachromatic effect occurs both at pH 6.1 and pH 7.0, and is independent of the buffering power of the reaction mixture, further demonstrating that the effect is not simply due to a change of pH in the bulk medium.

pH gradient-induced spectral shifts. The characteristics of the ATP-induced metachromatic effect mentioned above suggested that it is intimately associated with the ion transport properties of these membrane vesicles and the energization process of the membrane. We therefore tested the effects of an artificially imposed proton gradient from inside/out to energize the membranes (12). Fig. 4 shows the spectral shift of acridine orange upon an acid-base transition. Fig. 5 shows the time course of the spectral shift. The amount of spectral shift is dependent on the Δ pH. An acid stage of pH 6 produced only about one-half of the amount of shift

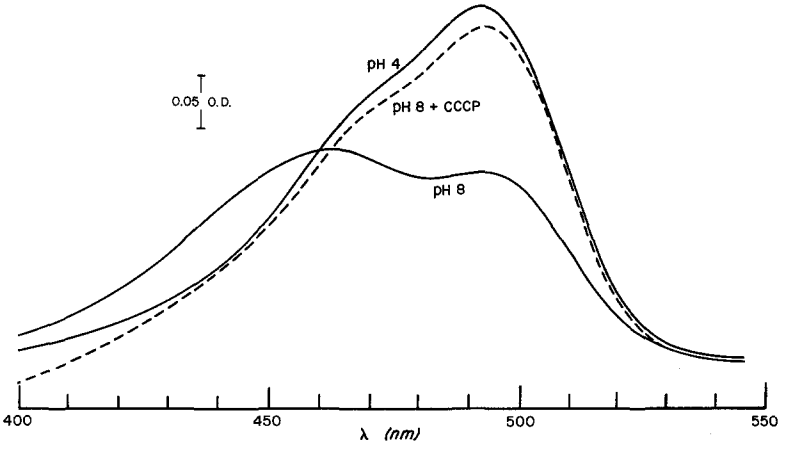

Fig. 4. Spectral changes induced by imposed pH gradient. Membrane vesicles (0.5 mg protein/ml) were incubated with 10 μM acridine orange and 10 mM succinate in K^+-Mg^{2+}-medium without PIPES at pH 4.0 for 1 min. The shifted spectrum was induced by adding 25 mM Tris-base to bring the medium to pH 8.0. The dotted spectrum shows the reversal induced by adding 2×10^{-5} M CCCP to the pH 8.0 spectrum.

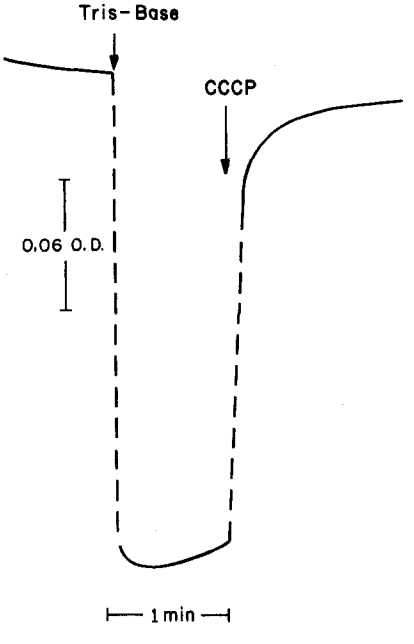

Fig. 5. Time course of the Δ pH induced spectral changes. Conditions are exactly the same as in Fig. 4 except that sample O.D. (493 nm-550 nm) was recorded as a function of time.

produced by pH 4 when the pH of the base stage was held at pH 8. K^+ gradient from inside/out severely inhibited the effect. Mg^{2+} at 2mM enhances the effect as compared with no Mg^{2+}. In the absence of K^+, valinomycin is ineffective in dissipating the effect; whereas CCCP alone can restore the O.D. to original level. However, in the presence of K^+, valinomycin is highly effective.

DISCUSSION The spectral shift induced by ATP is specific for ATP; it requires both Mg^{2+} and K^+, and it is inhibited by inhibitors of the ATPase. We therefore conclude that the spectral shift is closely related to the operation of the gastric microsomal K^+-ATPase. It has been shown that the operation of the K^+-ATPase is accompanied by an alkalization of the medium which has been interpreted as H^+ being pumped into the vesicles (3, 4 and insert of Fig. 1). Because of the response to ionophores, the spectral shift is most likely related to ion transport by these vesicles. The acid-base transition experiments indicate that an artificially imposed pH-gradient can also induce the spectral shifts of the dye. In view of the metachromatic property of the dye, the tentative conclusion we draw from this study is that as the membrane is energized either by ATP or a proton gradient, a conformational change takes place which produces changes in some anionic groups (11). The binding of dye molecules to these groups results in a "stacking phenomenon," similar to the binding of dye molecules to synthetic polyanions, which is responsible for the shift in the absorption spectrum.

The use of pH-electrode to measure ion transport is severely limited by the interference of pH changes produced when ATP is hydrolyzed to ADP and Pi (5, 6). Since the spectral shift is the result of the binding of the dye molecules to anionic sites in the membrane in response to energization of the membrane either by ATP or by pH gradient, and not simply the result of a pH change of the bulk medium, the ion transport properties of these membrane vesicles can be studied under a wide range of conditions by measuring

the metachromatic effect spectrophotometrically. Although we cannot quantitate the ions transported, the method is quite sensitive and may prove to be one of the ways to overcome the limitations of the pH-electrode method.

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