J. Biochem. 124, 547-556 (1998)

Prodigiosins Uncouple Mitochondrial and Bacterial F-ATPases: Evidence for Their H^+/Cl^- Symport Activity[1]

Hiroki Konno,* Hidekazu Matsuya,*,[2] Masayuki Okamoto,*,[3] Tomohiko Sato,* Yasufumi Tanaka,* Ken Yokoyama,* Takao Kataoka,† Kazuo Nagai,† Harry H. Wasserman,‡ and Shoji Ohkuma*,[4]

*Laboratory of Biochemistry, Department of Molecular and Cell Biology, Faculty of Pharmaceutical Sciences, Kanazawa University, Takara-machi 13-1, Kanazawa, Ishikawa 920-0934; †Department of Bioengineering, Tokyo Institute of Technology, Nagatsuta-chou 4259, Midori-ku, Yokohama, Kanagawa 226-0026; and ‡Department of Chemistry, Yale University, P.O. Box 208107, New Haven, Connecticut 06520-8107, USA

Received for publication, March 20, 1998

Prodigiosin, metacycloprodigiosin, and prodigiosin 25-C all inhibited the acidification activity of submitochondrial and bacterial (*Escherichia coli*) F-ATPases (F_oF_1-ATPases) strongly (IC_{50}=20-30 and 24-30 pmol/mg protein, respectively), without affecting significantly the ATP hydrolysis activity. Their effect on the acidification activity was rapid and reversible, showing non-competitive apparent K_i values of the order of nM to sub-nM. However, unlike FCCP (an ordinary uncoupler of oxidative phosphorylation), they showed no protonophoric activity, as demonstrated by the absence of acceleration of ATP hydrolysis. Prodigiosins also inhibited the acidification of proteoliposomes reconstituted from phospholipids and purified F-ATPase of *E. coli*, suggesting that their acidification-inhibitory effect is not due to the inhibition of anion channels. They did not, however, inhibit the ATP-dependent formation of membrane potential of F-ATPase vesicles. Furthermore, they inhibited and quickly reversed acidification by F-ATPase only in the presence of chloride, and not in the presence of gluconate anion. Finally, they induced swelling of liposomes and submitochondrial particles in isotonic solution of ammonium chloride but not ammonium gluconate, suggesting that intravesicular entry of Cl^- is promoted by prodigiosins. These results suggest that prodigiosins uncouple F-ATPases through promotion of H^+/Cl^- symport (or OH^-/Cl^- exchange) across vesicular membranes.

Key words: H^+-ATPase, F-ATPase, mitochondria, *Escherichia coli*, H^+/Cl^-symport.

Energy transduction in biological membranes is as important as signal transduction. In recent years, several important findings have been made regarding the mechanism of energy transduction in membranes (*1-3*), particularly in structural biology (*4-6*): X-ray crystallographic studies on bovine F_oF_1-ATPase (*4*) revealed structural interactions between subunits and supported a proposed rotational binding change model of H^+-transport triggered ATP synthesis (*7*), and these were followed by direct observation of the actual rotation of F_1 (*8*). Similarly, a proton translocation pathway has been proposed for the cytochrome oxidase complex, also based on X-ray crystallographic studies (*5, 6*). Nevertheless one important aspect of the model, namely, "energy coupling," remains to be clarified. The study of energy coupling can be approached in several ways, for example, in terms of molecular biology (mutagenesis) or biochemistry (using specific modifiers of energy transduction). From a biochemical point of view, specific modifiers of energy transduction would open the way to clarifying the energy transduction mechanism, just as uncouplers opened the way to confirming the chemiosmotic hypothesis proposed by Mitchell in 1961 (*9*).

Prodigiosins are candidates for such reagents: we reported previously that prodigiosin 25-C, a red pigment produced by microorganisms including *Streptomyces* and *Serratia*, uncouples lysosomal H^+-ATPase (V-ATPase) (*10*). Subsequently, we found that all the prodigiosins tested (prodigiosin, metacycloprodigiosin, and prodigiosin 25-C) inhibit the acidification mediated by lysosomal H^+-ATPase (V-ATPase), probably by facilitating the symport of H^+ and Cl^- (equivalent to OH^-/Cl^- exchange) and resulting in the uncoupling of acidification and ATP hydro-

[1] This work was supported by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

Present addresses: [2] Minase Research Inst., Ono Pharmaceutical Co., Ltd., Shimamoto, Osaka 618-0022; [3] Mie Res. Lab., Sanwa Kagaku Kenkyusho Co. Ltd., 363, Shiozaki, Hokusei, Inabe, Mie 511-0406. [4] To whom correspondence should be addressed. Tel: +81-76-234-4463, Fax: +81-76-234-4462, E-mail: ohkuma@kenroku.kanazawa-u.ac.jp

Abbreviations: BSA, bovine serum albumin; $\Delta\psi$, transmembrane potential gradient; ΔpH, transmembrane pH gradient; DCCD, *N*, *N'*-dicyclohexylcarbodiimide; DTT, dithiothreitol; EtOH, ethanol; FCCP, car- bonylcyanide *p*-trifluoromethoxyphenylhydrazone; F-ATPase, F-type H^+-ATPase; Hepes, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid; IMAC, mitochondrial inner membrane anion channel; NEM, *N*-ethylmaleimide; Oxonol-V, bis(3-phenyl-5-oxoisoxazol-4-yl)pentamethine oxonol; P-ATPase, P-type H^+-ATPase; SMP, submitochondrial particle; TBT, tributyltin chloride; TMAH, tetramethylammonium hydroxide; TMG, tritosomal membrane ghosts; TX-100, Triton X-100; V-ATPase, vacuolar type H^+-ATPase.

© 1998 by The Japanese Biochemical Society.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

lysis (and membrane potential formation) activities (Ohkuma *et al.*, manuscript in preparation).

In the present paper, we present evidence that prodigiosins also uncouple the acidification mediated by F-type H^+-ATPases (F-ATPases) of both submitochondrial and *Escherichia coli* inverted membrane vesicles from both ATP hydrolysis and membrane potential formation, in contrast to conventional uncouplers like FCCP that inhibit both acidification and membrane potential formation and stimulate ATP hydrolysis. Prodigiosins seem to uncouple F-ATPases through their activity to promote H^+/Cl^- symport across the membranes. But they do not inhibit ATPase activity, unlike TBT (a traditional OH^-/Cl^- exchanger), suggesting that they are a new group of H^+/Cl^- symporters that uncouple both V- and F-ATPases. A preliminary account of this work was given at the 22nd Meeting of the Japan Bioenergetics Group (Nagoya, 1996).

EXPERIMENTAL PROCEDURES

Materials—Rats (Wistar, male) were obtained from Sankyo Labo Service (Tokyo). *E. coli* strain DK8/pBWU13 (DK8 (*ΔuncB-C, ilv*::Tn 10), lacking the *unc* operon and harboring a recombinant plasmid pBWU13 (carrying the wild-type *unc* operon) (*11*), was supplied by Professor M. Futai (Institute of Scientific and Industrial Research, Osaka University). Prodigiosin was prepared from the culture broth of *Serratia marcescens* (HY-3) as described (*12*). Metacycloprodigiosin and prodigiosin 25-C were prepared from the culture broth of *Streptomyces hiroshimensis* as described previously (*13*). *E. coli* phospholipid was obtained from Avanti Polar Lipids (Alabaster, AL). *n*-Octyl-D-glucoside was purchased from Dojin Chemicals (Kumamoto), acridine orange was from Wako (Tokyo), and bis(3-phenyl-5-oxoisoxazol-4-yl)pentamethine oxonol (Oxonol-V) was from Nippon Kanko-Shikiso (Okayama). Other reagents were purchased as commercial products, mostly from Sigma (St. Louis, MO), and used without further purification, except that soybean phospholipid (a so-called 'asolectin') from Sigma (Type II-S) was further purified according to Newman (*14*) as described (*15, 16*).

Preparation of Mitochondria and Submitochondrial Particles (SMPs) from Rat Liver—Mitochondria and SMPs were prepared according to a published procedure (*17, 18*). Briefly, livers from rats (Wistar, male, 200-400 g in body weight, starved overnight) were homogenized (at 1,500 rpm, 2 strokes) with a Potter-Elvehjem type homogenizer in 220 mM mannitol, 70 mM sucrose, 2 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (Hepes)-KOH (pH 7.4), and 0.5 mg/ml bovine serum albumin (BSA), and centrifuged at $1,100\times g$ for 3 min. The precipitate was rehomogenized and recentrifuged. The combined supernatant was then centrifuged at $2,000\times g$ for 10 min, and the precipitate was resuspended and centrifuged at $3,000\times g$ for 3 min. The combined original and washed supernatant was again centrifuged at $2,000\times g$ for 15 min, and the fluffy layer was removed. The resultant precipitate was homogenized and centrifuged at $20,000\times g$ for 20 min. The precipitate (heavy mitochondrial fraction) was adjusted to 100 mg protein/ml and, after addition of digitonin (final 0.75%), incubated at 4°C for 15 min, diluted with 3 volumes of the same buffer, then centrifuged at $9,500\times g$ for 10 min. The precipitate was resuspended in distilled water, centrifuged under the same conditions, rehomogenized, centrifuged again at $9,500\times g$ for 15 min, then sonified for 15 s (8-cycles) at 50% duty in ice-water. The supernatant obtained after centrifugation at $9,500\times g$ for 10 min was again centrifuged at $210,000\times g$ for 30 min to obtain inverted membrane vesicles. These SMPs were resuspended in the isotonic buffer and stored at −80°C until use.

Preparation of Inverted Membrane Vesicles from E. coli—*E. coli* strain DK8/pBWU13 (*11*) grown in a rich medium (L broth) was cultured to late log phase in a minimal medium containing thymidine (50 μg/ml), thiamine (2 μg/ml), asparagine (50 μg/ml), isoleucine (50 μg/ml), valine (50 μg/ml), and 0.5% glycerol as the sole carbon source. Membrane vesicles were prepared by passing the cells (1 g, resuspended in 5 ml of TKDG buffer consisting of 10 mM Tris-HCl, pH 8.0, 140 mM KCl, 1 mM DTT, 10% glycerol) through a French press (1,500 kg/cm²), and centrifuging first at $12,000\times g$ for 10 min, and then the supernatant at $100,000\times g$ for 30 min. The resultant precipitate was resuspended in TKDG buffer, recentrifuged, and the resultant precipitate was resuspended in TKDG buffer at 20 mg/ml and stored at −80°C. This suspension was used as inverted membrane vesicles.

Purification and Reconstitution of E. coli H^+-ATPase into Proteoliposomes—Purification of *E. coli* H^+-ATPase and reconstitution of proton pump activity from purified H^+-ATPase into proteoliposomes was performed as described (*11*) with slight modifications. Briefly, the inverted membrane vesicles (40 mg protein/ml in TMEDG buffer consisting of 50 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 10% glycerol, 2 ml) were applied onto a stepwise sucrose density gradient consisting of 30, 35, 40, and 45.5% (w/w) sucrose and centrifuged at $112,000\times g$ for 16 h 20 min at 4°C. The 30-35% interface was collected and diluted in MMG buffer consisting of 5 mM MES-Tricine (pH 7.0), 2 mM $MgCl_2$, 5 mM monothioglycerol, then centrifuged at 4°C for 1 h. The precipitate was resuspended in MMG buffer at 40 mg/ml and used as the inner membrane. One milliliter of the inner membrane preparation was mixed with 20% (w/w) octylglucoside (final 0.8%) and centrifuged at $200,000\times g$ at 4°C for 1 h. The precipitate was resuspended in 1 ml of MMG buffer, mixed with 20% (w/w) of octylglucoside (final 2.0%), and recentrifuged at $200,000\times g$ at 4°C for 1 h. The resultant precipitate was applied onto the glycerol gradient (10-30% glycerol in 10 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes)-*N*-tris(hydroxymethyl)methylglycine (Tricine) (pH 7.0), 1% octylglucoside, 0.5 mM $MgCl_2$, and 5 mM monothioglycerol) and centrifuged at $330,000\times g$ at 4°C for 5 h. After centrifugation, fractions of 400-500 μl were collected from the bottom of the tube. Fractions 1-3 with the highest ATPase activity were combined and used as the purified F_oF_1.

The proton pump was reconstituted by the freeze-thaw-dilution method (*15*). Soybean phospholipid (Sigma type II-S, 10 mg dry weight) was suspended in 1 ml of 10 mM Mes-Tricine buffer (pH 7.0) containing 5 mM monothioglycerol and sonified under argon in a bath-type sonifier (Yamato) for 10 min. When the suspension became clear, it was stored at −20°C. F_oF_1-ATPase (150 μg protein, 180 μl) was mixed with 600 μg of the phospholipid (60 μl), frozen at −80°C, then thawed at 37°C and diluted with 10

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

mM Tricine-choline (pH 8.0), 0.1 M KCl, and 2 mM $MgCl_2$.

Proton Pump Assay—The acidification activity of inverted membrane vesicles was measured by means of the fluorescence quenching of acridine orange (*19*). The assay buffer contained 0.15 M KCl, 20 mM Hepes-TMAH (pH 7.5), and 2.5 mM $MgCl_2$ (with or without 1 μM valinomycin) for SMPs, and 0.14 M KCl, 10 mM Tricine-choline (pH 8.0), and 5 mM $MgCl_2$ with 1 μM valinomycin for inverted membrane vesicles of *E. coli*. The reaction was started by the addition of 2.5 mM (for SMPs) or 1.0 mM (for *E. coli* vesicles) ATP-Na_2 in a final volume of 2.0 ml. The concentration of acridine orange was either 1 or 2.5 μM. Fluorescence was measured with excitation at 480 (or 493) nm and emission at 540 (or 530) nm, respectively.

The formation of a membrane potential gradient (inside positive) was measured by the fluorescence quenching of Oxonol-V (*19*). The assay buffer (2 ml) contained 0.15 M KCl, 20 mM Hepes-TMAH (pH 7.5) for SMP, and 0.14 M KCl, 10 mM Tricine-choline (pH 8.0) for inverted membrane vesicles of *E. coli*. After addition of inverted membrane vesicles, 0.5–1 μM oxonol-V and 1 mM ATP-Na_2, the reaction was started by the addition of 5 mM $MgCl_2$, and the fluorescence measured with excitation at 580 nm and emission at 620 nm. For both measurements, the assay temperatures were 37 and 25°C for SMP and *E. coli* vesicles, respectively. The fluorescence was measured using a Hitachi 310 or F4500 spectrofluorometer, and the activities (acidification and membrane potential formation) were estimated from the initial velocity (defined as ΔF in 60 s) of ATP-dependent fluorescence quenching.

ATPase Assay—ATPase was assayed as described (*20*). The assay buffer contained 0.15 M KCl, 20 mM Hepes-TMAH (pH 7.5), 2.5 mM $MgCl_2$, and 2.5 mM ATP-Na_2 for SMPs, and 0.14 M KCl, 10 mM Tricine-choline (pH 8.0), and 5 mM $MgCl_2$ with 1 μM valinomycin for inverted membrane vesicles of *E. coli*, both in a final volume of 1.0 ml. The incubation temperature and period were 37°C and 40 min for SMP, and 25°C and 20 min for inverted vesicles of *E. coli*. The liberated phosphate was estimated spectrophotometrically by the Malachite Green method (*21*). All data shown are the averages (with deviation of less than 5%) of duplicated experiments.

Reversibility of Prodigiosin Action—For the preincubation, membrane vesicles were incubated in a total volume of 100 μl of solution containing valinomycin and acridine orange, with or without metacycloprodigiosin (10 nM) for 3 min at 37°C. After preincubation, 1.9 ml of assay buffer with or without metacycloprodigiosin (0.5 nM) was added, acidification was started by adding ATP (1 mM), and the change of acridine orange fluorescence was traced.

Preparation of Liposomes for Swelling Assay—Liposomes were prepared from *E. coli* phospholipid essentially as described (*15, 16*). Briefly, *E. coli* phospholipid was suspended (50 mg/ml) in 20 mM Hepes-TMAH (pH 7.5) containing 0.25 M sucrose (usually 2 ml), vortexed vigorously, and sonified briefly at room temperature.

Swelling Assay—The effect of prodigiosins on the permeability of mitochondrial and liposomal membranes to anions was evaluated by the swelling of mitochondria and liposomes in isotonic ammonium salts, which was monitored by the decrease in absorbance (turbidity) at 550 nm (A_{550}) (*22*). Briefly, mitochondria (heavy mitochondrial fraction) and liposomes were loaded at 0.8 mg protein/ml and 1 mg/ml, respectively, in 0.2 M solution (pH 7.0) of ammonium chloride (NH_4Cl) or ammonium gluconate (NH_4-gluconate), and after 30 s, prodigiosins (DMSO as solvent control) were added and A_{550} was monitored.

Other Analytical Methods—Protein was determined by the Amido Black/solid phase method of Schaffner and Weissman (*23*) or Coomassie Brilliant Blue/liquid phase method of Bradford (*24*) using a Bio-Rad protein assay kit according to the manufacturer's instructions (Bio-Rad, USA), with BSA as the standard.

RESULTS

Prodigiosins Inhibit Vesicular Acidification Driven by Mitochondrial F-ATPase—The effects of prodigiosin, metacycloprodigiosin, and prodigiosin 25-C on the acidification and ATPase activities of F-ATPases were examined. All three prodigiosins strongly and rapidly inhibited the acidification activity of submitochondrial H^+-ATPase (the results for metacycloprodigiosin are shown in Fig. 1). Figure 2A shows the dose response of the effect of the prodigiosins on the acidification and the ATPase activities of submitochondrial F-ATPase. The IC_{50}s of the inhibition of submitochondrial acidification were 2–3 nM under these conditions (at 100 μg protein/ml), corresponding to 20–30 pmol/mg protein for 50% inhibition. However, the prodigiosins did not inhibit ATP hydrolysis activity at concentrations up to 100 nM–1 μM, nearly 100 times higher than the IC_{50}s for acidification inhibition. At higher concentrations, however, they showed some inhibitory activities against ATPase activity with IC_{50}s of about 0.5–3 μM. On the other hand, ordinary proton channel inhibitors like *N,N'*-dicyclohexylcarbodiimide (DCCD) inhibited both

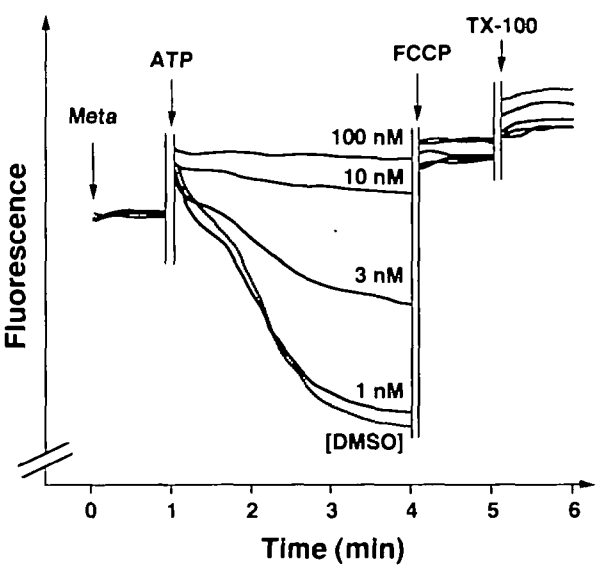

Fig. 1. **Effects of metacycloprodigiosin on the acidification activity of F-ATPase of SMP.** The acidification activity of SMP was estimated by the fluorescence quenching of acridine orange as described in "EXPERIMENTAL PROCEDURES." SMPs (about 200 μg protein) prepared from rat liver mitochondria were added to 2 ml of incubation mixture with or without the indicated concentration of metacycloprodigiosin (or 1% DMSO as solvent control) and incubated at 37°C for 1 min before the addition of ATP. DMSO, 1%; ATP, 1 mM ATP-Na_2; FCCP, 1 μM; TX-100, 0.1% Triton X-100.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

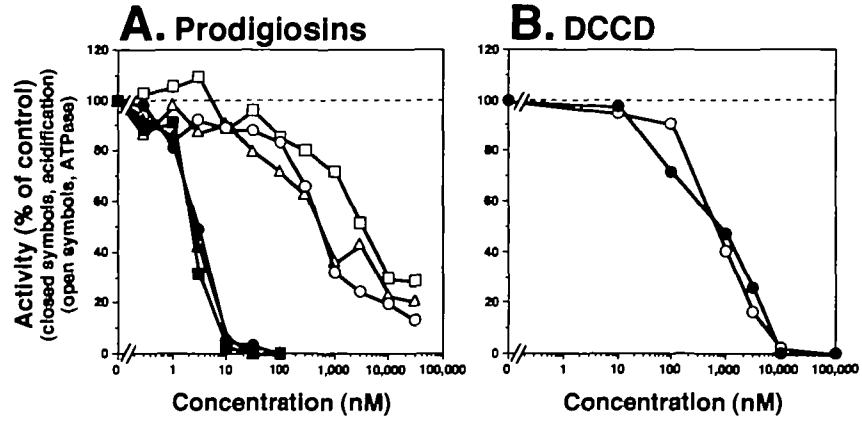

Fig. 2. **Effects of (A) prodigiosins as compared with (B) DCCD on the acidification and ATPase activities of F-ATPase of SMPs.** Acidification activity of the SMPs was measured as described in Fig. 1 in incubation mixture with or without the indicated concentrations of prodigiosin 25-C, metacycloprodigiosin, and prodigiosin (1% DMSO as solvent control) or DCCD (1% EtOH as solvent control). The SMPs (200 μg protein) were added 1 min before the addition of 1 mM ATP-Na_2. ATPase activity was measured as described under "EXPERIMENTAL PROCEDURES" in 1 ml of incubation mixture with or without the indicated concentrations of prodigiosin, metacycloprodigiosin, prodigiosin 25-C (1% DMSO as solvent control), or DCCD (1% EtOH as solvent control). The reaction was started by the addition of enzyme (0.63 μg protein) and the mixture was incubated for 20 min at 37°C. A: prodigiosins. (□, ■), prodigiosin; (○, ●), metacycloprodigiosin; (△, ▲), prodigiosin 25-C. B: DCCD. The specific Mg-ATPase activity of SMP was 1.67-1.78 U/mg protein.

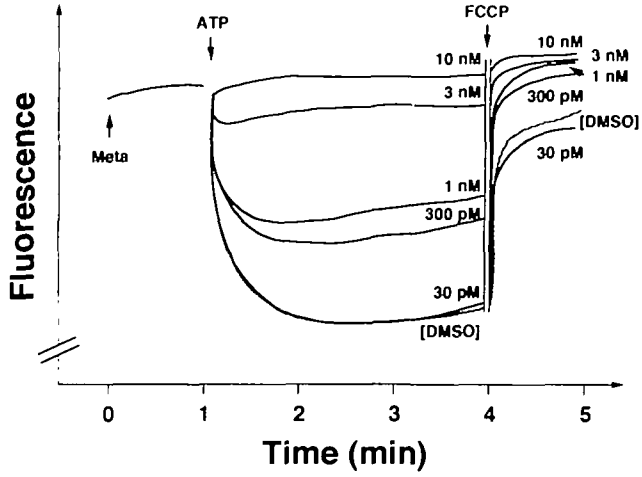

Fig. 3. **Effects of metacycloprodigiosin on the acidification activity of F-ATPase of the inverted membranes of *E. coli*.** The acidification activity of inverted membrane vesicles of *E. coli* was estimated as described under "EXPERIMENTAL PROCEDURES." Inverted membrane vesicles (10 μg protein) prepared from *E. coli* were added to 2 ml of incubation mixture with or without indicated concentrations of metacycloprodigiosin (or 1% DMSO as solvent control) and incubated at 37°C for 1 min before the addition of 1 mM ATP. ATP, 1 mM ATP-Na_2; Meta, metacycloprodigiosin; DMSO, 1%; FCCP, 1 μM.

acidification and ATP hydrolysis at similar concentrations with IC_{50} of 0.6 μM (Fig. 2B).

Prodigiosins Inhibit Vesicular Acidification Driven by Bacterial (E. coli) F-ATPase—Figure 3 shows the effect of metacycloprodigiosin on the acidification activity of F-ATPase on the inverted membrane vesicles of *E. coli*. All three prodigiosin antibiotics strongly and rapidly inhibited the acidification activity of inverted plasma membrane of *E. coli*. Figure 4 shows the dose response of the effect of these prodigiosins on the acidification and the ATPase activities of inverted plasma membranes of *E. coli*. The IC_{50}s of the inhibition of the acidification activity are 120-150 pM under these conditions (5 μg protein/ml, corresponding to 24-30 pmol/mg protein for 50% inhibition). However, the prodigiosins did not inhibit ATP hydrolysis activity up to concentrations of 0.1 μM, nearly 100 times higher than the IC_{50}s of acidification inhibition. At higher concentrations, they showed some inhibitory activities with IC_{50} of 0.3-1 μM. Again, ordinary proton channel inhibitors like DCCD inhibited both acidification and ATP hydrolysis at similar concentrations with IC_{50} of 30-10 μM. The slight dissociation between the two activities is partly due to the difference in the incubation conditions, especially the incubation period with DCCD, because DCCD requires some time for covalent binding with enzyme to exert its activity.

Prodigiosins, however, showed little protonophoric activity, unlike FCCP: uncoupling concentrations of prodigiosins (10 nM 25-C and 1 nM Meta) hardly activated (104-103% of control) the ATPase activities of SMP and *E. coli* vesicles, contrary to FCCP (2 μM) (182-162% of control).

Prodigiosins Reversibly and Non-Competitively Inhibit Acidification Activity of Inverted Membrane Vesicles Driven by F-ATPases—The rapid inhibitory effect of prodigiosins on the intravesicular acidification driven by F-ATPases was also completely reversible, as shown in Fig. 5. Inhibition of acidification activity of inverted membrane vesicles of *E. coli* by incubation with 10 nM metacycloprodigiosin was completely reversed by subsequent 50-fold dilution of the mixture to a final concentration of 0.2 nM metacycloprodigiosin. Essentially similar results have been obtained with SMP F-ATPase. Lineweaver-Burk plots of the inhibition by prodigiosins of the acidification of inverted membrane vesicles of mitochondria and *E. coli* showed that the inhibition was of a simple non-competitive type. Dixon plots gave single apparent K_i values of 1.2, 1.6, and 2.0 nM (at 100 μg protein/ml) (corresponding to 12-20 pmol/mg protein) for prodigiosin, metacycloprodigiosin, and prodigiosin 25-C for submitochondrial particles, and 120, 130, and 140 pM (at 5 μg protein/ml) (corresponding to 10-28 pmol/mg protein) for inverted membrane vesicles of *E. coli*, respectively.

Prodigiosin Inhibits Acidification of Proteoliposomes Reconstituted with Purified F-ATPase of E. coli—There are several ways of inhibiting vesicular acidification without inhibiting ATPase activity. One is to uncouple the H^+-ATPase molecules themselves; another is to block anion channels (*e.g.*, the chloride channel), which results in

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

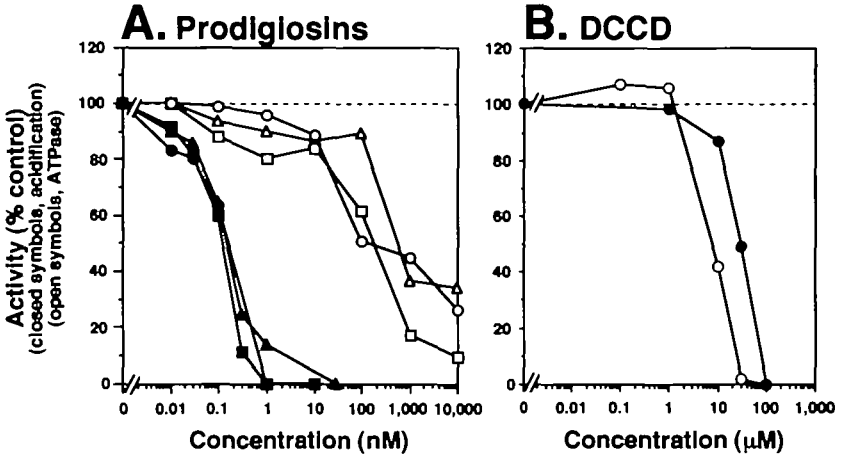

Fig. 4. **Effects of (A) prodigiosins as compared with (B) DCCD on the acidification and ATPase activities of F-ATPase of the inverted membranes of *E. coli*.** Acidification and ATPase activities of the inverted membrane vesicles of *E. coli* were measured as described under "EXPERIMENTAL PROCEDURES." For the acidification assay, inverted membrane vesicles (10 μg protein) prepared from *E. coli* were added to 2 ml of incubation mixture with or without the indicated concentration of prodigiosins (1% DMSO as solvent control) or DCCD (1% EtOH as solvent control) and incubated at 25°C for 1 min before the addition of ATP. The ATPase activity of *E. coli* vesicles (0.15 μg protein) was measured in 1 ml of incubation mixture with or without the indicated concentration of prodigiosins (1% DMSO as solvent control) or DCCD (1% EtOH as solvent control) and incubated at 25°C for 20 min. The reaction was started by the addition of 1 mM ATP-Na_2. A: prodigiosins. (□, ■), prodigiosin; (△, ▲), prodigiosin 25-C; (○, ●), metacycloprodigiosin. B: DCCD. The specific Mg-ATPase activity of inverted membrane vesicles of *E. coli* was 1.79 U/mg protein.

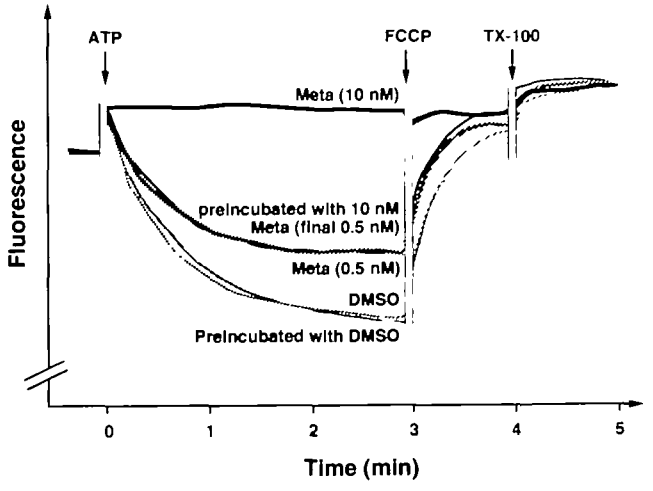

Fig. 5. **Reversibility of the effect of metacycloprodigiosin on the ATP-dependent acidification of inverted membrane vesicles of *E. coli*.** The inverted membrane vesicles (10 μg protein) of *E. coli* were added to 100 μl of preincubation mixture [10 mM Tricine-choline (pH 8.0), 0.14 M KCl, 5 mM $MgCl_2$ with or without 10 nM metacycloprodigiosin or 1% DMSO (solvent control)] and preincubated at 25°C for 2 min. Then 1.9 ml of dilution buffer [10 mM Tricine-choline (pH 8.0), 0.14 M KCl, 5 mM $MgCl_2$, and 1 μM acridine orange] was added to give a final concentration of 0.5 nM metacycloprodigiosin, and the mixture was incubated for 1 min before the addition of ATP. The acidification activity of membrane vesicles after each treatment was estimated by the fluorescence quenching of acridine orange as described under "EXPERIMENTAL PROCEDURES." ATP, 1 mM ATP-Na_2; Meta, metacycloprodigiosin; DMSO, 1%; FCCP, 2.5 μM; TX-100, 0.1% Triton X-100.

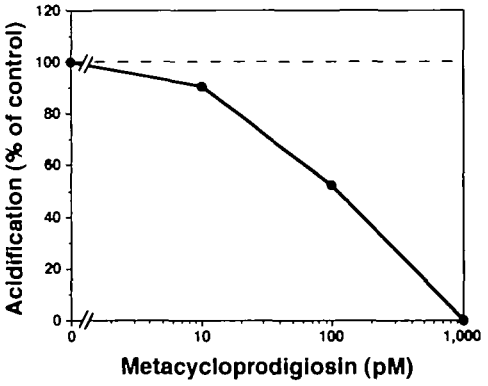

Fig. 6. **Effect of prodigiosin 25-C on the acidification activity of reconstituted F-ATPase.** Reconstituted proteoliposomes of *E. coli* F-ATPase were incubated at 25°C in an assay mixture containing 10 mM Tricine-choline (pH 8.0), 0.14 M KCl, 5 mM $MgCl_2$, 1 μM acridine orange, and the indicated concentration of metacycloprodigiosin (or 1% DMSO as solvent control). Acidification activity was measured as described in Fig. 3.

the inhibition of the compensatory movement of accompanying anions (like chloride) (to discharge membrane potential established by the H^+-ATPase), and therefore in the inhibition of continued acidification. To obtain direct evidence that the target molecule of prodigiosins is *not* anion channels, we performed a reconstitution experiment. The effect of prodigiosin on the acidification activity of the *E. coli* F-ATPase was measured in a reconstituted system (*11*). In this system, purified *E. coli* H^+-ATPase was solubilized and incorporated into liposomes made from asolectin through a freeze-thawing-sonication technique. As shown in Fig. 6, even acidification activity in this system was strongly inhibited, showing IC_{50} of about 10 pM. This result clearly shows that the target of prodigiosins is not anion channel(s) (*e.g.*, the inner membrane anion channel [IMAC (*25*)]).

Prodigiosins Do Not Inhibit ATP-Dependent Formation of Membrane Potential—Unlike ordinary inhibitors of the proton pump, prodigiosins did not inhibit the membrane potential ($\Delta\psi$) formation of F-ATPases. As shown in Fig. 7, the $\Delta\psi$ formation (inside positive) of inverted membrane vesicles of *E. coli* detected by the fluorescence quenching of oxonol-V was not affected by prodigiosins at concentrations ranging from 10 pM to almost 1 μM, while the ordinary uncoupler (protonophore) FCCP inhibited both acidification and $\Delta\psi$ formation equally well with IC_{50} of about 300 nM. The inhibition of $\Delta\psi$ formation by higher concentrations than 1 μM of prodigiosins is probably secondary to the inhibition of ATPase activity (see Figs. 2 and 4). Essen-

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

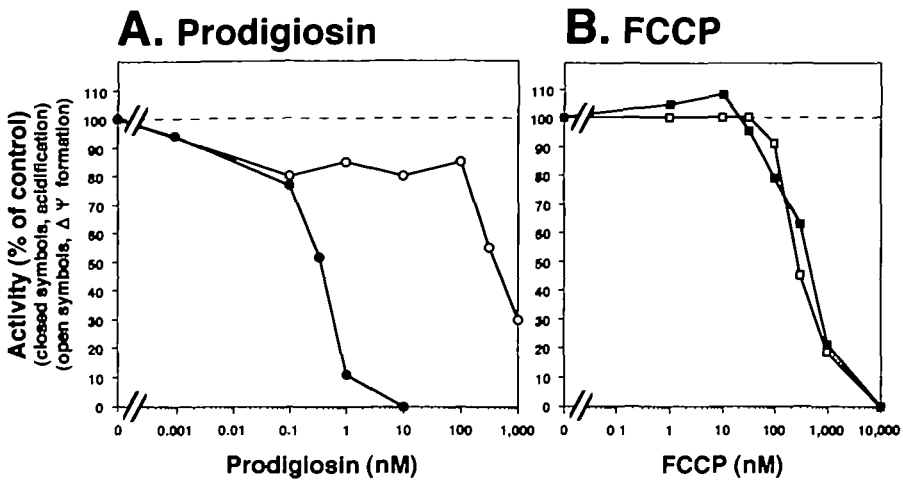

Fig. 7. **Effects of (A) prodigiosin as compared with (B) FCCP on the ATP-dependent formation of membrane potential.** ATP-dependent activities of acidification and membrane potential ($\Delta\psi$) formation of inverted membrane vesicles of *E. coli* were assayed as described under "EXPERIMENTAL PROCEDURES." The inverted membrane vesicles of *E. coli* (10 μg protein) were incubated for 1 min with or without the indicated concentration of prodigiosin (1% DMSO as solvent control) or FCCP (1% EtOH as solvent control) before the start of the assay. For both panels, closed symbols represent acidification and open symbols membrane potential ($\Delta\psi$) formation.

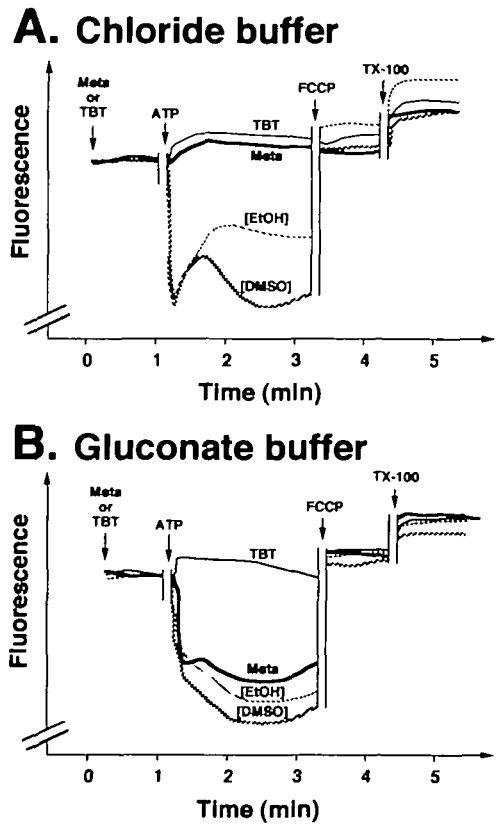

Fig. 8. **Anion requirement of the acidification inhibitory activity of metacycloprodigiosin on SMP.** Acidification activity was measured as described in Fig. 1, but in the presence of valinomycin. SMPs (200 μg protein) were added to 2 ml of incubation mixture [40 mM Hepes-TMAH (pH 7.5), 1 μM valinomycin and 2.5 μM acridine orange] containing (A) 0.15 M KCl or (B) 0.15 M K-gluconate, with metacycloprodigiosin (or 1% DMSO as solvent control) or with tributyltin chloride (TBT) (or 1% EtOH as solvent control) and incubated at 37°C for 1 min before the addition of 1 mM ATP-Mg. DMSO, 1%, EtOH, 1%; ATP, 1 mM ATP-Mg; Meta, 100 nM metacycloprodigiosin; TBT, 100 nM; FCCP, 1 μM; TX-100, 0.1% Triton X-100.

tially the same results were obtained with SMPs (data not shown).

There are also several ways of inhibiting vesicular acidification without inhibiting $\Delta\psi$ formation: firstly, (i) through inhibition of the transformation of ATP-driven $\Delta\psi$ into ΔpH (*26*) on the H^+-ATPase molecules themselves; secondly, (ii) through changing the ion specificity of the H^+-ATPase from H^+ to another cation (*e.g.*, K^+) [like Na-(V or F)-ATPase (*27-29*)]; or thirdly, (iii) through changing the ion specificity of the ATPase from H^+ (or OH^-) to another anion [like the relationship between halorhodopsin and bacteriorhodopsin (*30*) or OH^-/Cl^- exchangers (*22*)]. To test these possibilities, we next investigated the possible ion exchanging (or co-transporting) activity of prodigiosins on the F-ATPase membranes.

Prodigiosins Inhibit and Quickly Reverse Acidification Driven by F-ATPase Dependent on Chloride Ions—The inhibitory activity of prodigiosins on the vesicular acidification driven by F-ATPases was expressed *irrespective* of the cation species (data not shown). Therefore, we next looked into the anion requirement of the acidification inhibitory activity of prodigiosin. In Fig. 8, the inhibitory effect of prodigiosins on the acidification of SMPs was measured in buffers containing either chloride or gluconate as anion in the presence of valinomycin, where acidification was coupled with exchange extrusion of K^+. The acidification-inhibitory activity of prodigiosins was observed only in chloride buffer and not in gluconate buffer, suggesting a requirement of chloride ions for acidification-inhibitory activity. Figure 8 includes the results for tributyltin chloride (TBT), a well-known OH^-/Cl^- exchanger with potent H^+-ATPase inhibitory activity due to irreversible binding with the F_o portion of F- (and V-) ATPases (*31-38*): TBT inhibited acidification driven by F-ATPase both in chloride and gluconate buffers.

In Fig. 9, prodigiosins quickly reversed the acidification of the intravesicular pH driven by F-ATPase of *E. coli*, again only in chloride and not in gluconate buffer, without affecting seriously the membrane potential (data not shown). This was also the case with TBT. Essentially the same results were obtained with SMP (data not shown).

These results are consistent with the explanation that prodigiosins inhibit acidification due to their ability to mediate symport of H^+ with Cl^- or its equivalent anion exchange (OH^- with Cl^-).

Prodigiosins, Like TBT, Induce Cl^--Dependent Swelling

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

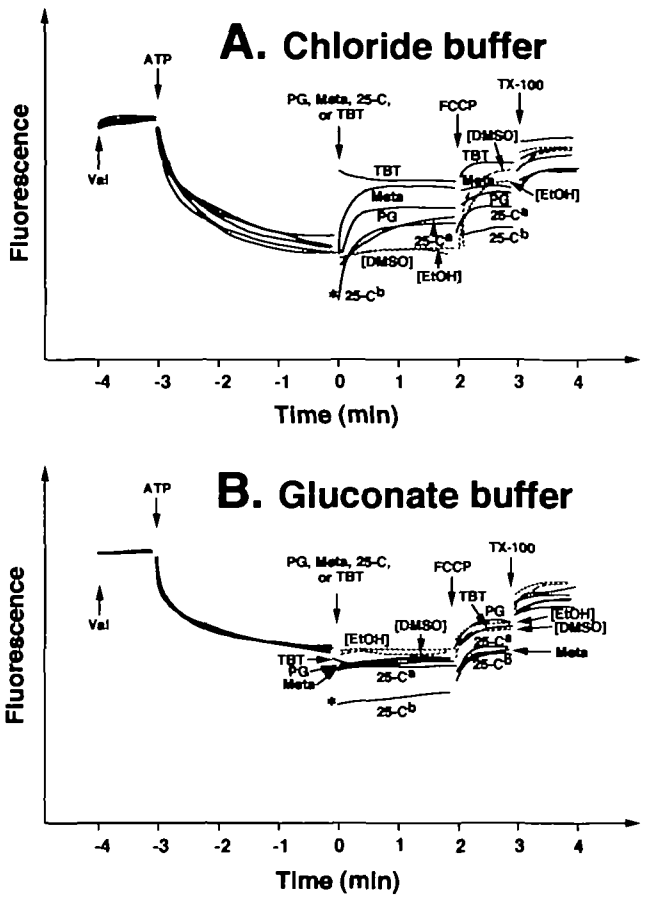

Fig. 9. **Prodigiosins quickly reverse the acidification of intravesicular pH of inverted membrane vesicles of *E. coli* in (A) chloride but not in (B) gluconate buffer.** Acidification activity was measured as described in Fig. 3, but in the presence of 0.1 μM valinomycin. Inverted membrane vesicles (10 μg protein) were added to 2 ml of incubation mixture [10 mM Tricine-choline (pH 8.0), and 2.5 μM acridine orange] containing (A) 0.14 M KCl or (B) 0.14 M K-gluconate and incubated at 37°C. One minute after the addition of 0.1 μM valinomycin, 1 mM ATP-Mg was added to drive vesicular acidification. After establishment of full acidification (at 4 min), the indicated concentrations of prodigiosins (prodigiosin, metacycloprodigiosin, and prodigiosin 25-C, 1% DMSO as solvent control) or TBT (1% EtOH as solvent control) were added to the incubation mixture. PG, 3 nM prodigiosin; Meta, 20 nM metacycloprodigiosin; 25-C^a, 40 nM prodigiosin 25-C; 25-C^b, 400 nM prodigiosin 25-C; DMSO, 1%; TBT, 20 nM; ATP, 1 mM ATP-Mg; Val, 0.1 μM valinomycin; FCCP, 1 μM; TX-100, 0.1% Triton X-100. *: The quick reduction of fluorescence after addition of the highest concentration (400 nM) of prodigiosin 25-C is due to the quenching effect on acridine orange fluorescence.

of Mitochondria and Liposomes—We finally tested if prodigiosins have this type of ionophoric activity on mitochondrial and liposomal membranes, using a well-established method to detect Cl^--dependent vesicular swelling in isotonic ammonium salt solution. In this method, anion permeability is estimated by vesicular swelling in isotonic solution of ammonium salts of anions of interest (*22*). As shown in Fig. 10, all the prodigiosins tested, like TBT, reduced the absorbance of mitochondria in dose- and time-dependent manner in 0.2 M NH_4Cl (but not in NH_4-gluconate): the effect of prodigiosin 25-C is weaker than the other prodigiosins and that of TBT is steeper in dose-response. Figure 11 represents the results with *E. coli* liposomes devoid of any protein constituents. The results are almost the same as those of mitochondria but the order of effectiveness was: TBT > metacycloprodigiosin = prodigiosin > prodigiosin 25-C. Essentially the same results have been obtained with asolectin liposomes (data not shown).

DISCUSSION

Although contrary to our original expectation, the data on the F-ATPases are consistent with the idea that the effects of prodigiosins are due to their ionophoric (H^+/Cl^- symporting) activity. One concern is that the acidification of F-type ATPase vesicles showed very high sensitivity to prodigiosins with lower IC_{50} and apparent K_i values (of the order of sub nM) than did lysosomal V-ATPases. But, this is most probably due to the lower concentrations of submitochondrial and *E. coli* membrane vesicle protein: when expressed on the basis of protein amount, the values are of almost the same order (10–100 pmol/mg protein for IC_{50}) for these experimental systems, suggesting that the target of prodigiosins is not a specific protein but non-specific membrane component, for example, phospholipids. Single K_i values do not necessarily indicate specific binding: in fact, the ionophore nigericin showed a single K_i value (approximately 1 nM at 220 μg protein/ml) of non-competitive inhibition of vesicular acidification driven by SMP H^+-ATPase (our unpublished observation). The other concern is the relatively weak activity of prodigiosin 25-C on liposomes. This might partly be due to the multilamellar nature of the liposomes used in this experiment, as the long alkyl side chain on prodigiosin 25-C may well retard its trans-multilayer movement. Otherwise, it might be a reflection of permeability differences (due to presence or absence of protein components) between liposomes and inverted membrane vesicles. The effect of TBT on liposomes is stronger than that of prodigiosins, which may reflect possible differences in their mode of action (*e.g.*, OH^-/Cl^- exchange *versus* H^+/Cl^- symport) and/or in their permeability properties.

Before deciding whether the above conclusion on the ionophoric activity of prodigiosins is valid, however, we should look into a completely different proton translocation system, for example, P-type ATPases (whose proton translocation is mediated by a phosphorylated intermediate, unlike F- or V-type H^+-ATPase), respiratory proton pumps, vacuolar membrane pyrophosphatases, and so on. We are now investigating the effect and precise mode of action of prodigiosins on these proton pumps, as well as on the phospholipid vesicles.

The findings and conclusions described in the present article explain, first of all, why the prodigiosins did not affect cellular ATP content although they inhibit mitochondrial H^+-ATPase (F-ATPase) (*10*). As they do not inhibit $\Delta\psi$ formation of H^+-ATPases, they are not expected to affect the proton motive force driven by Mg-ATP in H^+-ATPase. In fact, this has been the case with the traditional hydroxyl/chloride ion exchangers, tributyltin or other trialkyl(organo)tin compounds on the generation of proton motive force by respiration: TBT does not affect $\Delta\psi$ formation of cytochrome *c* oxidase (*39*) and therefore does not affect the formation of the proton-motive force (nor,

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

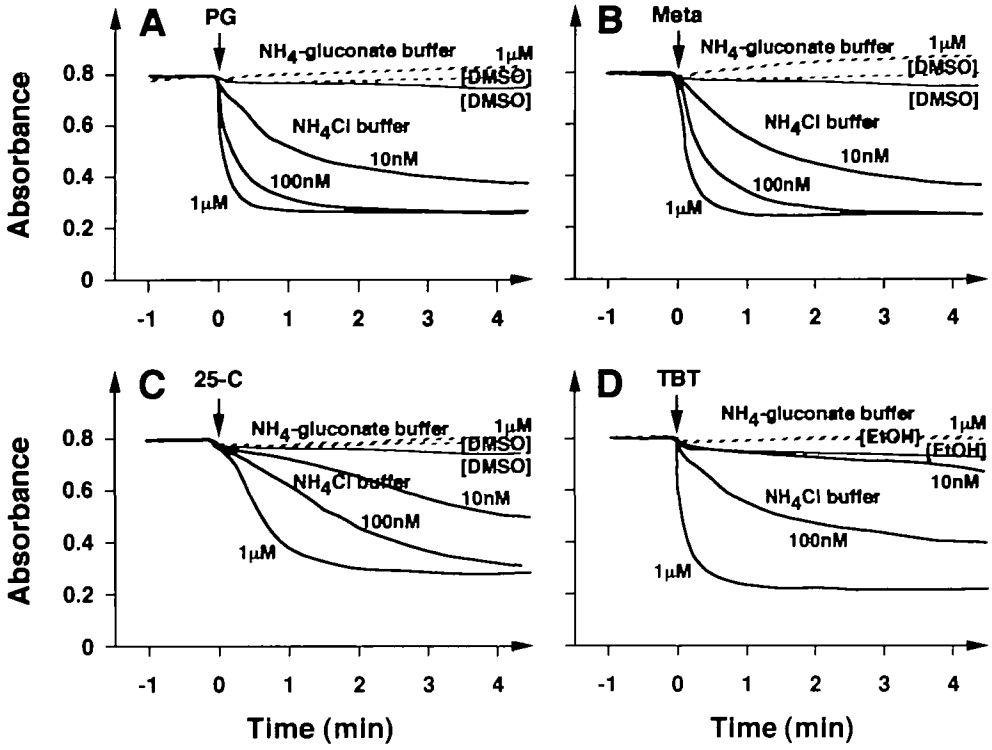

Fig. 10. **Cl^--dependent swelling of mitochondria by prodigiosins.** Mitochondria (heavy mitochondrial fraction) were loaded at 0.8 mg protein/ml in 0.2 M solution (pH 7.0) of ammonium chloride (NH_4Cl) or ammonium gluconate (NH_4-gluconate), and the A_{550} was monitored. A, prodigiosin; B, metacycloprodigiosin; C, prodigiosin 25-C; D, TBT. PG, prodigiosin; Meta, metacycloprodigiosin; 25-C, prodigiosin 25-C; [DMSO], 1% DMSO (solvent control for prodigiosins); [EtOH], 1% EtOH (solvent control for TBT). Solid lines, NH_4Cl buffer; dotted lines, NH_4-gluconate buffer.

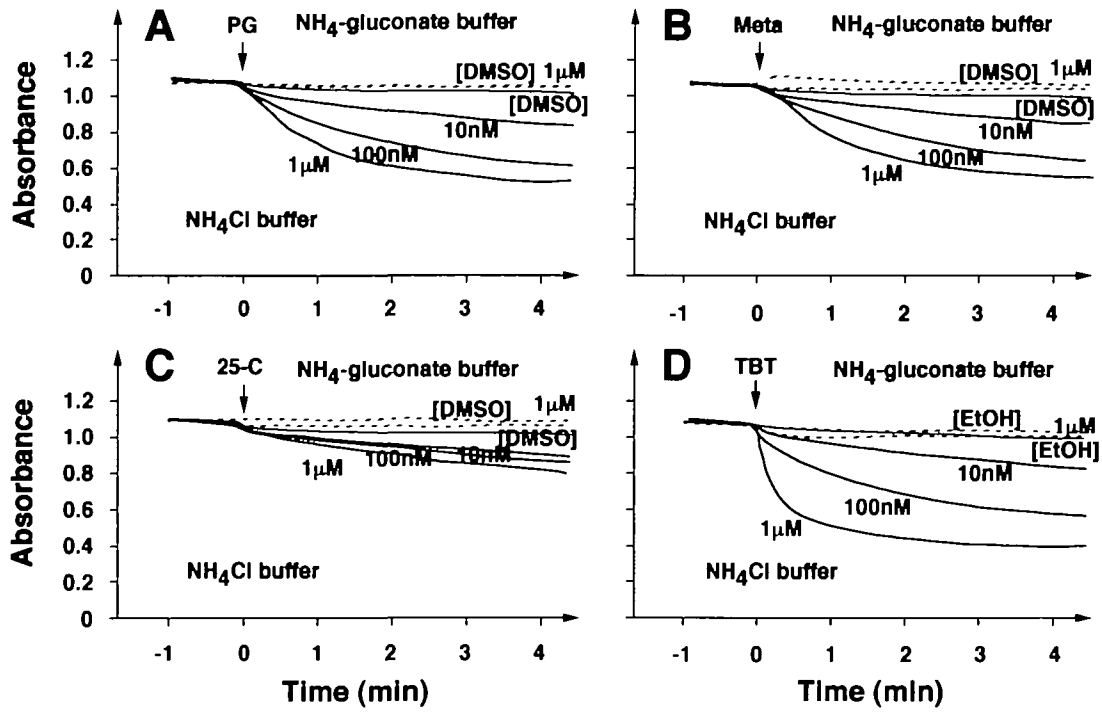

Fig. 11. **Cl^--dependent swelling of liposomes by prodigiosins.** Liposomes prepared from *E. coli* phospholipids were added at 1 mg/ml to 0.2 M solution (pH 7.0) of ammonium chloride (NH_4Cl) or ammonium gluconate (NH_4-gluconate), and the A_{550} was monitored. A, prodigiosin; B, metacycloprodigiosin; C, prodigiosin 25-C; D, TBT. PG, prodigiosin; Meta, metacycloprodigiosin; 25-C, prodigiosin 25-C; [DMSO], 1% DMSO (solvent control for prodigiosins); [EtOH], 1% EtOH (solvent control for TBT). Solid lines, NH_4Cl buffer; dotted lines, NH_4-gluconate buffer.

probably, ATP formation) driven by respiration. In fact, TBT has been used as such a reagent (*40*). However, trialkyltins bind F- (and even V-) ATPases and inhibit their ATPase and ATP synthetic activities (*33, 41-44*). We will show that prodigiosins, although they inhibit respiration-induced acidification, do not inhibit respiration (and there-

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

fore, $\Delta\psi$ formation) of mitochondria and of *E. coli* (manuscripts in preparation). Secondly, these characteristics also explain the mitochondrial swelling (*10*): the anion exchanging property of TBT coupled with the internal alkalinization (plus inside negative membrane potential) of *living* mitochondria driven by the respiratory proton pump will drive the uptake of osmotically active chloride anions [through IMAC (*25*)], resulting in the accumulation of osmotically active chloride salts (*e.g.*, KCl), which will eventually produce mitochondrial swelling (*41, 45-47*).

Prodigiosins behave as H^+/Cl^- symporters (or OH^-/Cl^- exchangers), but they inhibit acidification only and not ATPase activity, in contrast with the conventional OH^-/Cl^- exchanger TBT, which inhibits both acidification and ATPase activities with IC_{50} of 5-15 nM and 150 nM, respectively (our unpublished data). Therefore, prodigiosins may constitute a new group of H^+/Cl^- symporters (or OH^-/Cl^- exchangers) which will be useful for the study of the biological reactions involving H^+ (or OH^-) and/or Cl^-. At higher concentrations (approximately 1 μM), however, prodigiosins also inhibited the ATP hydrolysis activities of both inverted membrane vesicles of mitochondria (SMP) and *E. coli* with IC_{50} of 0.5-3 and 0.3-1 μM, respectively. These results indicate that prodigiosins have some affinity for H^+-ATPases, possibly to binding site(s) for lipophilic cationic dyes (*48*). We are now investigating the mode of inhibition of ATPase activity by prodigiosins.

We are grateful to Prof. M. Futai (Institute of Industrial Sciences, Osaka University) for the generous gift of *E. coli* strain DK8 harboring a recombinant plasmid pBWU13 (carrying the wild-type *unc* operon). We are also grateful for the excellent technical assistance of Mr. T. Shigeoka, Ms. K. Hirano, Ms. A. Kojima, and Ms. A. Hayashi (Faculty of Pharmaceutical Sciences, Kanazawa University). We thank Dr. K. Arai (Faculty of Pharmaceutical Sciences, Kanazawa University) for invaluable discussions, and Prof. Emeritus Y. Anraku (Graduate School of Science, The University of Tokyo) for critical reading of the manuscript.

REFERENCES

1. Pedersen, P.L. and Amzel, L.M. (1993) ATP synthases: Structure, reaction center, mechanism, and regulation of one of nature's most unique machines. *J. Biol. Chem.* **268**, 9937-9940
2. Ferguson, S.J. *et al.* (1995) Mitochondrial and bacterial ATPases. *Biochem. Soc. Trans.* **23**, 735-789
3. Moller, J.V., Juul, B., and le Maire, M. (1996) Structural organization, ion transport, and transduction of P-type ATPases. *Biochim. Biophys. Acta* **1286**, 1-51
4. Abrahams, J.P., Leslie, A.G.W., Lutter, R., and Walker, J.E. (1994) Structure at 2.8 Å resolution of F_1-ATPase from bovine heart mitochondria. *Nature* **370**, 621-628
5. Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995) Structure at 28 Å resolution of cytochrome *c* oxidase from *Paracoccus denitrificans*. *Nature* **376**, 660-669
6. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1995) Structures of metal sites of oxidized bovine heart cytochrome *c* oxidase at 2.8 Å. *Science* **269**, 1069-1074
7. Boyer, P.D. (1993) The binding change mechanism for ATP synthase: some probabilities and possibilities. *Biochim. Biophys. Acta* **1140**, 215-250
8. Noji, H., Yasuda, R., Yoshida, M., and Kinoshita, K., Jr. (1997) Direct observation of the rotation of F_1-ATPase. *Nature* **386**, 299-302
9. Mitchell, P. (1961) Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature* **191**, 144-148
10. Kataoka, T., Muroi, M., Ohkuma, S., Waritani, T., Magae, J., Takatsuki, A., Kondo, S., Yamazaki, M., and Nagai, K. (1995) Prodigiosin 25-C uncouples vacuolar type H^+-ATPase, inhibits vacuolar acidification and affects glycoprotein processing. *FEBS Lett.* **359**, 53-59
11. Moriyama, Y., Iwamoto, A., Hanada, H., Maeda, M., and Futai, M. (1991) One-step purification of *Escherichia coli* H^+-ATPase (F_oF_1) and its reconstitution into liposomes with neurotransmitter transporters. *J. Biol. Chem.* **266**, 22141-22146
12. Wasserman, H.H., McKeon, J.E., Smith, L.A., and Forgione, P. (1966) Studies on prodigiosin and the bipyrrole precursor. *Tetrahedron* Suppl. **8**, Part II, 647-662
13. Harashima, K., Tsuchida, N., Tanaka, T., and Nagatsu, J. (1967) Prodigiosin-25 C: isolation and the chemical structure. *Agric. Biol. Chem.* **31**, 481-489
14. Newman, M.J. and Wilson, T.H. (1980) Solubilization and reconstitution of the lactose transport system from *Escherichia coli*. *J. Biol. Chem.* **255**, 10583-10586
15. Kasahara, M. and Hinkle, P.C. (1977) Reconstitution and purification of the D-glucose transporter from human erythrocytes. *J. Biol. Chem.* **252**, 7384-7390
16. Sase, S., Anraku, Y., Nagano, M., Osumi, M., and Kasahara, M. (1982) Random distribution of the glucose transporter of human erythrocytes in reconstituted liposomes. *J. Biol. Chem.* **257**, 11100-11105
17. Bustamante, E., Soper, J.W., and Pedersen, P. (1977) A high-yield preparative method for isolation of rat liver mitochondria. *Anal. Chem.* **80**, 401-408
18. Wehrle, J.P., Cintrón, N.M., and Pedersen, P.L. (1978) Phosphate transport in rat liver mitochondria. *J. Biol. Chem.* **253**, 8598-8603
19. Moriyama, Y., Takano, T., and Ohkuma, S. (1984) Proton translocating ATPase in lysosomal membrane ghosts: evidence that alkaline Mg^{2+}-ATPase acts as a proton pump. *J. Biochem.* **95**, 995-1007
20. Arai, K., Shimaya, A., Hiratani, N., and Ohkuma, S. (1993) Purification and characterization of lysosomal H^+-ATPase: an anion-sensitive v-type ATPase from rat liver lysosomes. *J. Biol. Chem.* **268**, 5649-5660
21. Chan, K.-M., Delfert, D., and Junger, K.D. (1986) A direct colorimetric assay for calcium-stimulated ATPase activity. *Anal. Biochem.* **157**, 375-380
22. Selwyn, M.J., Dawson, A.P., Stockdale, M., and Gains, N. (1970) Chloride-hydroxide exchange across mitochondrial, erythrocyte and artificial lipid membranes mediated by trialkyl- and triphenyltin compounds. *Eur. J. Biochem.* **14**, 120-126
23. Schaffner, W. and Weissmann, C. (1973) A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal. Biochem.* **56**, 502-514
24. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72**, 248-254
25. Beavis, A.D. (1992) Properties of the inner membrane anion channel in intact mitochondria. *J. Bioenerg. Biomembr.* **24**, 77-90
26. Higuti, T. (1993) Function of mitochondrial H^+-pumps as a molecular parallel condenser in *New Functionality Materials: Design, Preparation and Control* (Tsuruta, T., Seno, M., and Doyama, M., eds.) pp. 131-138, Elsevier Science Publishers, B.V., Amsterdam
27. Kalm, G. and Dimroth, P. (1995) A double mutation in subunit *c* of the Na^+ specific F_1F_o-ATPase of *Propionigenium modestum* results in a switch from Na^+ to H^+-coupled ATP synthesis in the *Escherichia coli* host cells. *J. Mol. Biol.* **253**, 726-738
28. Kakinuma, Y., Kakinuma, S., Takase, K., Konishi, K., Igarashi, K., and Yamato, I. (1993) A gene encoding the 16-kDa proteolipid subunit of *Enterococcus hirae* Na^+-ATPase complex. *Biochem. Biophys. Res. Commun.* **195**, 1063-1069
29. Udagawa, T., Unemoto, T., and Tokuda, H. (1986) Generation of Na^+ electrochemical potential by the Na^+-motive NADH oxidase and Na^+/H^+ antiport system of a moderately halophilic *Vibrio costicola*. *J. Biol. Chem.* **261**, 2616-2622

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012

30. Sasaki, J., Brown, L.S., Chon, Y.-S., Kandori, H., Maeda, A., Needleman, R., and Lanyi, J.K. (1995) Conversion of bacteriorhodopsin into a chloride ion pump. *Science* **269**, 73-75
31. Dawson, A.P., Farrow, B.G., and Selwyn, M.J. (1982) Studies on the nature of the high-affinity trialkyltin binding site of rat liver mitochondria. *Biochem. J.* **202**, 163-169
32. Hughes, G., Harrison, M.A., Kim, Y.-I., Griffiths, D.E., Finbow, M.E., and Findlay, J.B.C. (1996) Interaction of dibutyltin-3-hydroxyflavone bromide with the 16 kDa proteolipid indicates the disposition of proton translocation sites of the vacuolar ATPase. *Biochem. J.* **317**, 425-431
33. Usta, J. and Griffiths, D.E. (1993) Dibutyltin-3-hydroxyflavone bromide: a fluorescent inhibitor of F_1F_o-ATPase. *Appl. Organometal. Chem.* **7**, 193-200
34. Farrow, B.G. and Dawson, A.P. (1978) Investigation of the interaction of triethyltin with rat liver mitochondria using binding studies and Mössbauer spectroscopy. *Eur. J. Biochem.* **86**, 85-95
35. Cain, K. and Griffiths, D.E. (1977) Studies of energy-linked reactions: localization of the site of action of trialkyltin in yeast mitochondria. *Biochem. J.* **162**, 575-580
36. Gould, J.M. (1976) Inhibition by triphenyltin chloride of a tightly-bound membrane component involved in photophosphorylation. *Eur. J. Biochem.* **62**, 567-575
37. Singh, A.P. and Singh, K. (1984) Isolation and characterization of tributyltin resistant mutants of *Escherichia coli*. *Z. Naturforsch.* **39c**, 293-299
38. Barranco, J., Darszon, A., and Gómez-Puyou, A. (1981) Extraction of mitochondrial protein-lipid complexes into organic solvents: inhibition of cytochrome oxidase electron transport by dicyclohexylcarbodiimide and triphenyltin chloride. *Biochem. Biophys. Res. Commun.* **100**, 1402-1408
39. Singh, A.P. and Nicholls, P. (1986) Effects of triorganotin-mediated anion-hydroxide exchange upon reconstituted cytochrome *c* oxidase proteoliposomes. *Biochem. Cell Biol.* **64**, 647-655
40. Mukohata, Y. and Kaji, Y. (1981) Light-induced membrane-potential increase, ATP synthesis, and proton uptake in *Halobacterium halobium* R1mR catalyzed by Halorhodopsin: effects of *N,N'*-dicyclohexylcarbodiimide, triphenyltin chloride, and 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile (SF6847). *Arch. Biochem. Biophys.* **206**, 72-76
41. Stockdale, M., Dawson, A.P., and Selwyn, M.J. (1970) Effects of trialkyltin and triphenyltin compounds on mitochondrial respiration. *Eur. J. Biochem.* **15**, 342-351
42. Aldridge, W.N. (1976) The influence of organotin compounds on mitochondrial functions. *Adv. Chem. Ser.* **157**, 186-196
43. Selwyn, M. (1976) Triorganotin compounds as ionophores and inhibitors of ion translocating ATPases. *Adv. Chem. Ser.* **157**, 204-226
44. Emanuel, E.L., Carver, M.A., Solani, G.C., and Griffiths, D.E. (1984) Differential inhibition of F_oF_1-ATPase-catalized reactions in bovine heart submitochondrial particles by organotin compounds. *Biochim. Biophys. Acta* **766**, 209-214
45. Wulf, R.G. and Byington, K.H. (1975) On the structure-activity relationships and mechanism of organotin induced, nonenergy dependent swelling of liver mitochondria. *Arch. Biochem. Biophys.* **167**, 176-185
46. Wakashin, K. (1975) Experimental studies on the toxicity of organic tin compounds—morphology of the butyl-tin intoxication. *Tokyo Ikadaigaku Zasshi* **33**, 573-596
47. Yoshizuka, M., Hara, K., Haramaki, N., Yokoyama, M., Mori, N., Doi, Y., Kawahara, A., and Fujimoto, S. (1992) Studies on the hepatotoxicity induced by bis(tributyltin)oxide. *Arch. Toxicol.* **66**, 182-187
48. Bullough, D.A., Ceccarelli, E.A., Roise, D., and Allison, W.S. (1989) Inhibition of the bovine-heart mitochondrial F_1-ATPase by cationic dyes and amphipathic peptides. *Biochim. Biophys. Acta* **975**, 377-383

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on October 1, 2012