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Stoichiometry of metal–tetracycline/H^+ antiport mediated by transposon Tn10-encoded tetracycline resistance protein in *Escherichia coli*

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The tetracycline resistance protein (TetA) endoded by transposon Tn10 mediates the efflux of divalent cation–tetracycline chelating complexes [Yamaguchi, A., Udagawa, T. and Sawai, T. (1990) J. Biol. Chem. 265, 4809–4813]. It was confirmed that protons were antiported with the complexes through an electrically-neutral process because the antiport consumed ΔpH but not $\Delta\psi$. The quantitative relationship between ΔpH and ΔpTC determined by a flow-dialysis method clearly indicated a 1:1 stoichiometry of the monocationic metal–tetracycline/H^+ exchange.

Tetracycline/H^+ antiporter; Tetracycline; Stoichiometry; Tetracycline resistance protein; Antiport; Flow dialysis

1. INTRODUCTION

The tetracycline resistance encoded by transposon Tn10 is based on the efflux of the drug [1]. The efflux is driven by a proton motive force and is thought to involve an antiport with protons [1, 2]. We reported that the antiport is electrically neutral because (i) the tetracycline uptake by inverted membrane vesicles prepared from *E. coli* cells producing the tetracycline resistance protein (TetA) was inhibited by the K^+/H^+ antiporter, nigericin, but not by the K^+ ionophore, valinomycin, and (ii) the tetracycline uptake by the inverted vesicles was driven by an artificial ΔpH but not by an artificial $\Delta\psi$ [3]. On the other hand, tetracycline molecules are present as a neutral or monoanionic form under physiological conditions [4]. If such a neutral or monoanionic molecule is antiported with protons, the process should be electrogenic and driven by $\Delta\psi$. This obvious conflict between experimental evidence and the theoretical conclusion was resolved by our discovery that the substance pumped out is not the free tetracycline molecule but a monocationic complex of tetracycline and a divalent cation [5]. However, there has been no direct evidence that protons are really antiported by TetA and, if they are, it is not known whether or not the stoichiometry of the antiport is 1:1, as expected from the electrically-neutral model. Recently, we first succeeded in detecting protons antiported with tetracycline using quinacrine as a fluorescent indicator [6].

In this paper, the properties of the proton antiport with tetracycline, mediated by TetA, were investigated using a ΔpH indicator, quinacrine, and a $\Delta\psi$ indicator, oxonol V. Then the stoichiometry of the exchange of the metal–tetracycline complex with protons was determined. In principle, the stoichiometry can be determined in 2 different ways: (i) as the ratio of the initial rates of tetracycline and proton transport, and (ii) as the relationship between the concentration gradients of tetracycline and protons across the membrane in equilibrium. The latter can be more accurately determined than the former. In this study, the stoichiometry was determined by measuring the tetracycline and proton gradients across energized membrane vesicles by means of a flow-dialysis method.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

E. coli W3104 [7] was used for the preparation of inverted vesicles. pOT3 (R388::Tn10) [8] was kindly provided by T. Iino.

2.2. *Materials*

Quinacrine and oxonol V (NK-2047) were purchased from Sigma and Nippon Kankoh-shikiso Kenkyusho Co., Okayama, Japan, respectively. [^{14}C]methylamine and [7-^{3}H]tetracycline were purchased from New England Nuclear. Other chemicals were commercial products of the highest grade available.

2.3. *Preparation of inverted membrane vesicles*

E. coli W3104/pOT3 cells were grown in a minimal medium supplemented with 0.2% glucose and 0.1% casamino acids in the presence of 4 μg/ml of tetracycline. At the mid-log phase, the cells were harvested and washed once with 0.1 M phosphate buffer (pH 6.6) or 50 mM MOPS-KOH buffer (pH 6.6) containing 10 mM EDTA, and then resuspended in the same buffer. Then, after the addition of DNase II (40 μg/ml), the cells were disrupted with a French press (Amicon) under 5000 psi. Undisrupted cells were removed by centrifugation at 15 000 rpm and then the vesicles were collected by ultracentrifugation at 36 000 rpm. The vesicles were washed once with 50 mM potassium phosphate buffer (pH 7.5) or 50 mM MOPS-KOH (pH 7.5), and then suspended in the same buffer and stored at −80°C.

2.4. *Measurement of the fluorescence of quinacrine and oxonol V*

ΔpH and $\Delta\psi$ across the inverted membrane vesicles were monitored as the changes in the fluorescence of quinacrine [9] and oxonol V [10], respectively. A suspension (100 µl) of inverted vesicles (2 mg protein/ml; prepared and suspended in MOPS-KOH buffer as described above) was diluted with 2 ml of 50 mM MOPS-KOH (pH 7.5) and 10 mM $MgSO_4$. After the addition of 10 µl of a quinacrine (169 µM) or oxonol V (200 µM) solution, the fluorescence emission of quinacrine was measured at 500 nm with excitation at 440 nm, and that of oxonol V at 635 nm with excitation at 580 nm. Five µl of 250 mM NADH, 5 µl of 2 mM tetracycline and 2 µl of 10 mM CCCP were successively added at about 2 min intervals.

2.5. *Determination of ΔpH and ΔpTC by flow dialysis*

Flow dialysis was performed as described by Tokuda and Unemoto [11]. Inverted membrane vesicles (10 mg protein; prepared and suspended in phosphate buffer as described above) suspended in 0.4 ml of 50 mM potassium phosphate (pH 7.5) containing 10 mM $MgSO_4$, in the presence or absence of 2 µg/ml valinomycin, were put into the upper chamber of a flow cell and then dialysed against a continuous flow of 50 mM phosphate buffer (pH 7.5) in the lower chamber, which was separated from the upper chamber by a cellulose membrane. The flow rate was 1 ml per min. The solutions in both the upper and lower chambers were stirred with a magnetic stirrer. [^{3}H]Tetracycline (30 µM) or [^{14}C]methylamine (236 µm) was added to the vesicle suspension in the upper chamber and the radioactivity of the buffer flowing out from the lower chamber was continuously monitored with a Flow-one radioactivity monitor (Radiomatic Instruments and Chemical Co., Tampa, FL, USA) using a liquid scintillator (flow rate; 4 ml per min) for ^{3}H or a solid scintillator for ^{14}C. Counts accumulated in one min were printed out. The concentrations of the radioactive compounds in the vesicles were calculated taking the inner volume of the vesicles to be 1 µl/mg protein [12]. At the times indicated, 20 mM (final) ascorbate and various concentrations of phenazine methosulfate (PMS) (ranging from 1 to 50 µM) were simultaneously added, and after about 20 min, 20 µM CCCP was added.

3. RESULTS AND DISCUSSION

3.1. *The effects of proton antiport with tetracycline on ΔpH and $\Delta\psi$ across inverted vesicles*

ΔpH and $\Delta\psi$ across inverted membrane vesicles prepared from *E. coli* cells carrying transposon Tn10 were monitored as to fluorescence changes of quinacrine and oxonol V, respectively. When the vesicles were energized by the addition of NADH, the fluorescence of both quinacrine and oxonol V decreased because ΔpH and $\Delta\psi$ were generated through respiration (Fig. 1). The generation of ΔpH was slower than that of $\Delta\psi$. When 5 µM tetracycline was added to the energized vesicles, the quinacrine fluorescence increased (Fig. 1A), indicating the abolition of ΔpH. The tetracycline-induced fluorescence increase in quinacrine was direct evidence that protons were antiported with tetracycline by TetA. It is clear that the protons were antiported with a divalent cation-tetracycline chelating complex because the fluorescence of quinacrine was not changed by tetracycline in the absence of a divalent cation (data not shown). In addition, when CCCP was added to the energized vesicles, the fluorescence of quinacrine returned to the same level whether tetracycline and divalent cations were added or not, confirming that the tetracycline-induced fluorescence change was due to the

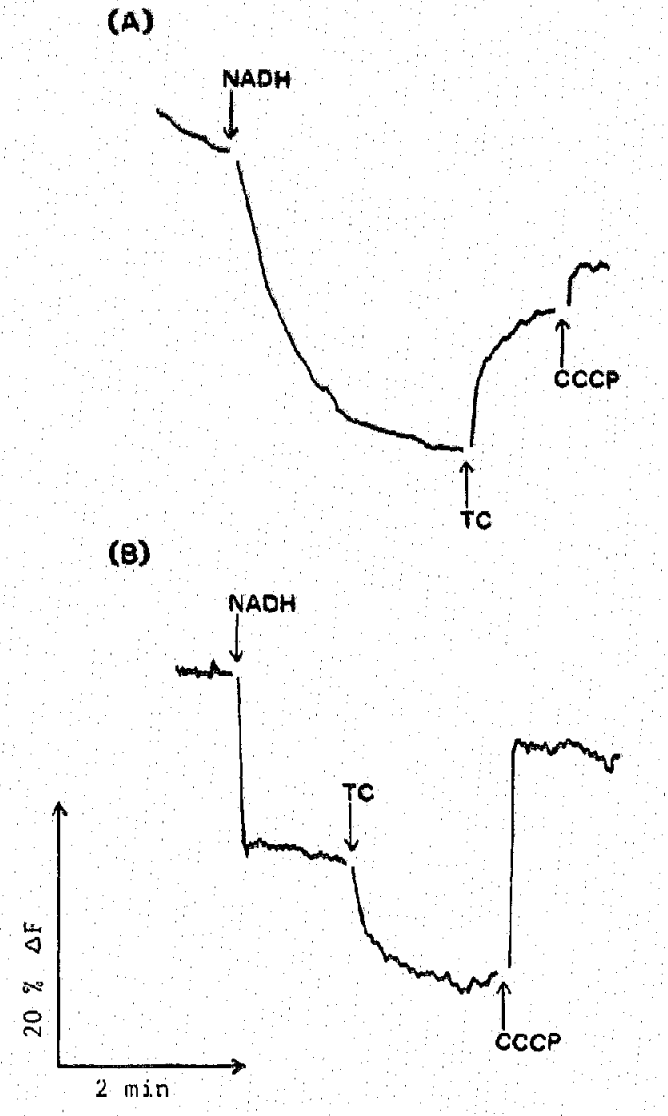

Fig. 1. The effects of proton antiport with tetracycline on ΔpH and $\Delta\psi$. ΔpH (A) and $\Delta\psi$ (B) across inverted membrane vesicles prepared from *E. coli* cells carrying transposon Tn10 were monitored for the fluorescence changes of quinacrine and oxonol V, respectively. Arrows indicate the times of addition of the reagents indicated.

abolition of ΔpH generated by respiration. The change in fluorescence caused by tetracycline did not occur in vesicles prepared from cells carrying no plasmid (data not shown).

On the other hand, in contrast to quinacrine, oxonol V fluorescence was further decreased by the addition of 5 µM tetracycline (Fig. 1B). A decrease in the fluorescence of oxonol V also did not occur in the absence of a divalent cation (data not shown), indicating that the decrease was due to divalent cation-tetracycline/H^+ antiport. Therefore, it can be concluded that the antiport does not consume $\Delta\psi$ but rather increases $\Delta\psi$. This phenomenon is not plausible if the antiport is driven by $\Delta\psi$. In contrast, it is reasonable to consider

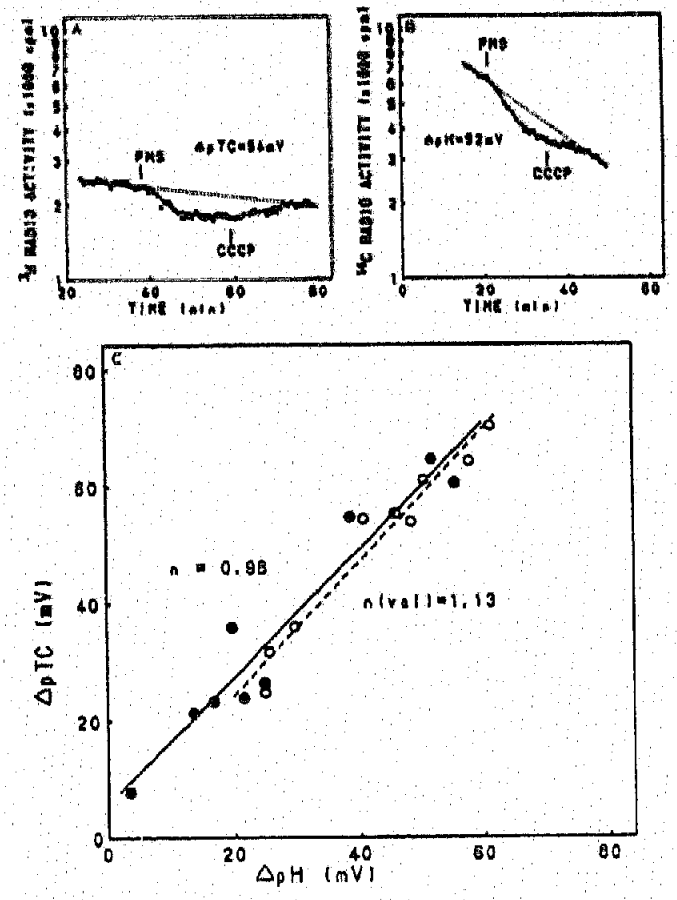

Fig. 2. Quantitative relationship between ΔpTC and ΔpH across inverted vesicles prepared from *E. coli* cells carrying transposon Tn10. (A and B) Typical results of flow dialysis using [^{3}H]tetracycline (for ΔpTC) and [^{14}C]methylamine (MA) (for ΔpH), respectively, when 50 μM PMS (final) was added. Arrows indicate the times of addition of the reagents indicated. Ascorbate was added simultaneously with PMS. ΔpTC and ΔpH calculated from these data are shown in the figure. ΔpTC (mV) = 58.8 log([TC]$_{in}$/[TC]$_{out}$) and ΔpH (mV) = 58.8 log([MA]$_{in}$/[MA]$_{out}$). (C) Plot of ΔpTC vs ΔpH. ΔpTC and ΔpH were calculated from the results of a number of flow dialysis experiments, when various concentrations of PMS were added. Each plot represents the average for duplicate experiments. Closed circles with a solid line and open circles with a broken line represent the plots in the absence and presence of valinomycin, respectively. n and n(val) indicate the slopes of the plots in the absence and presence of valinomycin, respectively, the slopes being calculated by means of linear least squares regression.

that the consumption of one component, ΔpH, of the proton motive force by the electrically-neutral antiport causes the shift of the proton motive force to another component, $\Delta\psi$. Thus, the tetracycline-induced decrease in oxonol V fluorescence is strong evidence for the electrically-neutral nature of the tetracycline/H^+ antiport.

3.2. *Quantitative relationship between ΔpH and ΔpTC across inverted vesicles*

When inverted membrane vesicles prepared from *E. coli* cells carrying transposon Tn10 were energized with 20 mM ascorbate and various concentrations of PMS, the concentration gradients of tetracycline and protons generated across the inverted vesicles were measured, using a ΔpH indicator, [^{14}H]methylamine, and [^{3}H]tetracycline, respectively, by means of a flow-dialysis method. Typical flow-dialysis data are shown in Fig. 2A and B, when 50 μM PMS was used. ΔpTC (= 58.8 log([TC]$_{in}$/[TC]$_{out}$)) and ΔpH (= 58.8 log([H^+]$_{in}$/[H^+]$_{out}$)) calculated from the data of Fig. 2A and B were 54 mV and 52 mV, respectively.

When n mols of protons are antiported with one mol of tetracycline, the equilibrium of the antiport reaction is:

$$[TC]_{out} + n[H^+]_{in} \rightleftharpoons [TC]_{in} + n[H^+]_{out}$$

The equilibrium constant, K_{eq}, can be represented as follows:

$$K_{eq} = \frac{[TC]_{in}[H^+]^n_{out}}{[TC]_{out}[H^+]^n_{in}}$$

The equation can be rearranged to:

$$\log \frac{[TC]_{in}}{[TC]_{out}} = n \log \frac{[H^+]_{in}}{[H^+]_{out}} + \log K_{eq}$$

Thus, the slope of the plot of ΔpTC vs ΔpH represents the stoichiometry, n. The plot of ΔpTC vs ΔpH is shown in Fig. 2C. Each plot was the average of 2 independent experiments. The slope was equal to 0.96, indicating that the stoichiometry is 1. When $\Delta\psi$ was abolished by the addition of valinomycin, there was no significant difference in the ΔpTC vs ΔpH plot, confirming the electrically-neutral nature of the antiport. The slope in the presence of valinomycin was equal to 1.13, which is roughly equal to 1 within experimental error.

The experimental evidence presented in this paper clearly indicates a 1 : 1 antiport model of a monocationic complex of a divalent cation and a tetracycline molecule with a proton molecule (Fig. 3).

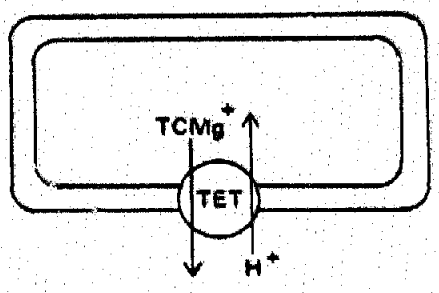

Fig. 3. A 1 : 1 exchange model of the metal-tetracycline/H^+ antiport in *E. coli* cells. $TCMg^+$, monocationic complex of a tetracycline molecule and a magnesium ion.

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