pH-Dependent Changes in Proton:Substrate Stoichiometries during Active Transport in *Escherichia coli* Membrane Vesicles†

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ABSTRACT: Experiments are presented in which the proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$) in *Escherichia coli* membrane vesicles (interior negative and alkaline) was measured under a variety of conditions and compared with steady-state levels of accumulation of lactose, proline, D-lactate, and glucose-6-P measured under identical conditions. Accumulation of lactose and proline is proportional to the magnitude of $\Delta\bar{\mu}_{H^+}$ at pH 5.5, where the pH gradient (ΔpH) and the electrical potential ($\Delta\Psi$) both contribute to $\Delta\bar{\mu}_{H^+}$, and at pH 7.5, where $\Delta\Psi$ represents the only component of $\Delta\bar{\mu}_{H^+}$. Moreover, the proportionality constants between $\Delta\bar{\mu}_{H^+}$ and lactose or proline accumulation indicate that the proton:substrate stoichiometries are 1:1 at pH 5.5 and 2:1 at pH 7.5. Evidence is also presented which indicates that the functional group responsible for the increase in proton:proline stoichiometry has a pK of approximately 6.8. Accumulation of D-lactate and glucose-6-P is directly related to the magnitude of ΔpH at pH 5.5, and stoichiometry values of one and approximately 1.7 are obtained for D-lactate and glucose-6-P, respectively, at this pH. At pH 7.5, on the other hand, accumulation of each organic acid bears a linear relationship to $\Delta\Psi$, and proton:substrate stoichiometries of unity are observed in both instances. The results are consistent with the models discussed by Rottenberg (Rottenberg, H. (1976), *FEBS Lett. 66,* 159).

Isolated bacterial membrane vesicles retain the same polarity as the membrane in the intact cell and catalyze active transport of many solutes by a respiration-dependent mechanism that does not involve the generation of utilization of ATP or other high-energy phosphate compounds (Kaback, 1974). Recent studies with this system provide almost unequivocal confirmation of the hypothesis that chemiosmotic phenomena, as postulated by Mitchell (1961, 1966, 1968, 1973; Harold, 1972), play a central, obligatory role in this process (Ramos et al., 1976; Kaback, 1976; Ramos and Kaback, 1977a,b; Tokuda and Kaback, 1977). In vesicles prepared from *Escherichia coli* and *Salmonella typhimurium,* oxidation of D-lactate or reduced phenazine methosulfate (PMS)[1] leads to the development of an electrochemical gradient of protons across the membrane (interior negative and alkaline) which is composed of interconvertible electrical and chemical parameters according to the following relationship:

$$\Delta\bar{\mu}_{H^+} = \Delta\Psi - \frac{2.3RT}{F}\Delta\text{pH} \qquad (1)$$

where $\Delta\bar{\mu}_{H^+}$ represents the electrochemical gradient of protons, $\Delta\Psi$ denotes the electrical potential across the membrane, and ΔpH is the chemical difference in proton concentrations across the membrane ($2.3RT/F$ is equal to 58.8 mV at room temperature).

Addition of transport substrates such as lactose or glucose-6-P which are accumulated in relatively large amounts by the appropriate vesicles results in partial collapse of $\Delta\Psi$ (Schuldiner and Kaback, 1975) and/or ΔpH (Ramos and Kaback, 1977b), providing direct evidence for the proposition that respiratory energy can drive active transport via the $\Delta\bar{\mu}_{H^+}$ across the membrane. Titration studies with valinomycin and nigericin (Ramos and Kaback, 1977b) indicate that, at pH 5.5, there are two general classes of transport systems: those that are coupled preferentially to $\Delta\bar{\mu}_{H^+}$ (lactose, proline, serine, glycine, tyrosine, glutamate, leucine, lysine, cysteine, and succinate) and those that are coupled preferentially to ΔpH (glucose-6-P, lactate, glucuronate, and gluconate). It is eminently clear, however, that, at pH 7.5, all of these transport systems are driven by $\Delta\Psi$ which comprises the only component of $\Delta\bar{\mu}_{H^+}$ at this external pH (Ramos et al., 1976; Ramos and Kaback, 1977a,b). In addition, when the effect of external pH on the steady-state level of accumulation of various transport substrates is examined, none of the pH profiles corresponds to those observed for $\Delta\bar{\mu}_{H^+}$, ΔpH, or $\Delta\Psi$. Furthermore, at external pH values exceeding 6.0–6.5, $\Delta\bar{\mu}_{H^+}$ is insufficient thermodynamically to account for the concentration gradients observed for most of the substrates if it is assumed that the stoichiometry between protons and solute remains constant at 1:1. These findings and the observation that the accumulation of organic acids is coupled to $\Delta\Psi$ at relatively high external pH have led to the suggestion that the stoichiometry between protons and transport substrates may increase at relatively high external pH (Ramos et al., 1976; Rottenberg, 1976; Kaback, 1976; Ramos and Kaback, 1977b). In this paper, additional experimental support for this argument is presented.

Experimental Section

Methods

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* ML 308-225 ($i^-z^-y^+z^+$) was grown on 1.0% disodium succinate (hexahydrate) and *E. coli* GN-2 ($i^-z^+y^+a^+$ enzyme I^-) on 0.2% disodium glucose-6-P, and membrane vesicles were prepared as described previously (Ramos and Kaback, 1977a).

For studies at various pHs, membrane vesicles which had been prepared and frozen in 0.1 M potassium phosphate (pH 6.6) were thawed and transferred to 0.1 M potassium phos-

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[1] Abbreviations used: PMS, phenazine methosulfate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DMO, 5,5-dimethyloxazolidine-2,4-dione; $TPMP^+$, triphenylmethylphosphonium.

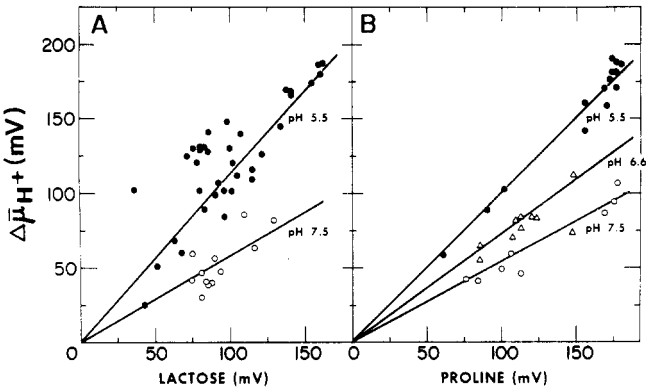

FIGURE 1: Relationship between $\Delta\bar{\mu}_{H^+}$ and steady-state levels of lactose (panel A) and proline (panel B) accumulation at various external pH values. Membrane vesicles prepared from *E. coli* ML 308-225 grown on succinate (Methods) were transferred into 0.1 M potassium phosphate at pH 5.5 (●—●), pH 6.6 (△—△), and pH 7.5 (○—○) as described in Methods. Aliquots (0.4 mL containing about 6 mg of membrane protein) were diluted to a final volume of 0.8 mL in the upper chamber of a flow dialysis apparatus. All reaction mixtures contained (in final concentrations) 0.05 M potassium phosphate (pH 5.5, 6.6, or 7.5, as indicated), 0.01 M magnesium sulfate, 12 μM $TPMP^+$, 37.5 μM sodium acetate (or 200 μM DMO), and 0.4 mM lactose (A) or 14 μM proline (B). In some experiments, valinomycin (in concentrations ranging up to 5 μM), nigericin (in concentrations ranging up to 0.1 μM), or CCCP (in concentrations ranging up to 5 μM) was also added to the reaction mixture in the upper chamber, and in other experiments, valinomycin (5 μM) plus nigericin (in concentrations ranging up to 0.1 μM) were added. Under all conditions, flow dialysis was performed as described (Ramos et al., 1976; Ramos and Kaback, 1977a,b; Tokuda and Kaback, 1977) using sodium ascorbate (20 mM, final concentration) and PMS (0.1 mM, final concentration) as electron donor. For measurements of $\Delta\Psi$, [^{3}H]$TPMP^+$ (4.36 Ci/mmol) was used in place of unlabeled $TPMP^+$. For measurements of ΔpH at pH 5.5 and 6.6, sodium [1,2-^{14}C]acetate (54 mCi/mmol) or [2-^{14}C]DMO (11 mCi/mmol) was used in place of unlabeled sodium acetate or DMO. For measurements of steady-state levels of lactose (A) and proline (B) accumulation, [1-^{14}C]lactose (60 mCi/mmol) and [U-^{14}C]proline (260 mCi/mmol) were used in place of unlabeled lactose and proline. It should be emphasized that each experimental point represents the results of flow dialysis determinations in which ΔpH, $\Delta\Psi$, and lactose or proline accumulation were measured under identical conditions.

phate at the desired pH as described (Ramos et al., 1976; Ramos and Kaback, 1977a,b). The protein concentration of the suspensions was adjusted to approximately 6.0 mg per mL.

Transport Assays. All transport data presented in this paper were derived from flow dialysis assays (Ramos et al., 1976; Ramos and Kaback, 1977a,b; Tokuda and Kaback, 1977). Determinations were performed with the upper chamber of the apparatus open to the atmosphere, and the reaction mixtures were gassed with oxygen. The upper and lower chambers were separated by Spectrapor 1 dialysis tubing (6000–8000 molecular weight cut-off; Fisher Scientific), and both chambers were stirred with magnetic bars. Membrane vesicles suspended in 0.05 M potassium phosphate at a given pH containing 0.01 M magnesium sulfate were added to the upper chamber (total volume 0.8 mL), and sodium ascorbate and PMS were used at final concentrations of 20 and 0.1 mM, respectively. Ionophores and isotopically labeled solutes were used as indicated. The same buffer (0.05 M potassium phosphate at the same pH as the upper chamber) was pumped from the lower chamber at a rate of 6.0 mL per min using a Pharmacia pump (Model P3). Fractions of 1.7 mL were collected and assayed for radioactivity by liquid scintillation spectrometry.

Since ascorbate–PMS was used as electron donor in all of these experiments, the increase in external pH of the upper chamber was monitored and taken into account in calculations of ΔpH (Ramos and Kaback, 1977a). It is also noteworthy in this regard that the increase in external pH with time causes some diminution in the steady-state level of accumulation of the solutes used after 3–5 min. Thus, all steady-state concentration gradients were calculated from initial equilibrium values obtained after addition of ascorbate and PMS (cf. Figure 1 in Ramos and Kaback, 1977a).

Determination of ΔpH. ΔpH was determined by assaying the accumulation of [1,2-^{14}C]acetate or 5,5-dimethyl[2-^{14}C]oxazolidine-2,4-dione (DMO) using flow dialysis (Ramos et al., 1976; Ramos and Kaback, 1977a; Tokuda and Kaback, 1977), and the increase in external pH induced by the oxidation of ascorbate was taken into account in the calculations (Ramos and Kaback, 1977a).

Determination of $\Delta\Psi$. The electrical potential across the membrane ($\Delta\Psi$) was determined by measuring the accumulation of [^{3}H]triphenylmethylphosphonium ($TPMP^+$) (bromide salt) using flow dialysis (Schuldiner and Kaback, 1975; Ramos et al., 1976; Ramos and Kaback, 1977a,b; Tokuda and Kaback, 1977).

Calculations. Concentration gradients for solutes taken up by the vesicles were calculated using a value of 2.2 μL of intravesicular fluid per mg of membrane protein (Kaback and Barnes, 1971). Internal pH was calculated as described in Waddel and Butler (1959) and Schuldiner et al. (1972), and ΔpH was determined by difference. The electrical potential ($\Delta\Psi$) was calculated from the Nernst equation ($\Delta\Psi = 58.8 \log [TPMP^+]_{in}/[TPMP^+]_{out}$). The proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$) was calculated by substituting values for ΔpH and $\Delta\Psi$ into eq 1. According to the chemiosmotic formulation (Mitchell, 1961, 1966, 1968, 1973; Harold, 1972), active transport occurs by proton:substrate symport, and it is the proton movements that couple transport to $\Delta\bar{\mu}_{H^+}$, ΔpH, or $\Delta\Psi$. Thus, in order to relate solute accumulation to $\Delta\bar{\mu}_{H^+}$, ΔpH, and $\Delta\Psi$, concentration ratios for lactose, proline, D-lactate, and glucose-6-P were converted into millivolts (mV) using the Nernst equation (mV = 58.8 log concentration gradient).

Protein Determinations. Protein was measured as described by Lowry et al. (1951) using bovine serum albumin as a standard.

Materials

[^{3}H]Triphenylmethylphosphonium bromide was prepared by the Isotope Synthesis Group at Hoffmann-La Roche, Inc., under the direction of Dr. Arnold Liebman as described (Schuldiner and Kaback, 1975). Other isotopically labeled materials were purchased from New England Nuclear and Amersham/Searle. Valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were obtained from Calbiochem. Nigericin was the generous gift of Dr. J. Berger of Hoffmann-La Roche Inc.

Results

The data presented in Figure 1 were derived from experiments in which $\Delta\bar{\mu}_{H^+}$ was determined under a variety of conditions and, in parallel experiments, compared with steady-state levels of accumulation of lactose (Figure 1A) and proline (Figure 1B) measured under the same conditions. As shown previously (Ramos and Kaback, 1977b), transport of these two solutes is coupled preferentially to $\Delta\bar{\mu}_{H^+}$ at pH 5.5. It is clear from these data that accumulation of lactose and proline is proportional to the magnitude of $\Delta\bar{\mu}_{H^+}$ over a wide range of values at pH 5.5 where ΔpH and $\Delta\Psi$ both contribute to $\Delta\bar{\mu}_{H^+}$ and at pH 7.5 where $\Delta\Psi$ represents the totality of the driving force (Ramos et al., 1976; Ramos and Kaback, 1977a). Moreover, to a best approximation, the relationship between $\Delta\bar{\mu}_{H^+}$ and lactose or proline accumulation exhibits a slope of

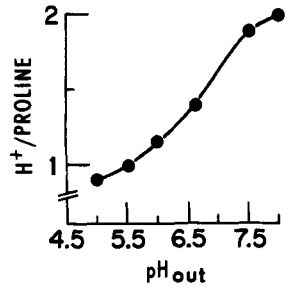

FIGURE 2: Change in proton:proline stoichiometry as a function of external pH. Each experimental point shown in the figure is derived from a series of flow dialysis experiments in which ΔpH, ΔΨ, and steady-state levels of proline accumulation were measured under identical conditions (i.e., in the absence or presence of valinomycin, nigericin, CCCP, or valinomycin plus nigericin) at each of the external pH values given as described in Figure 1. The data obtained at each external pH were then plotted as shown in Figure 1, and the stoichiometry was calculated from the inverse slope of each function.

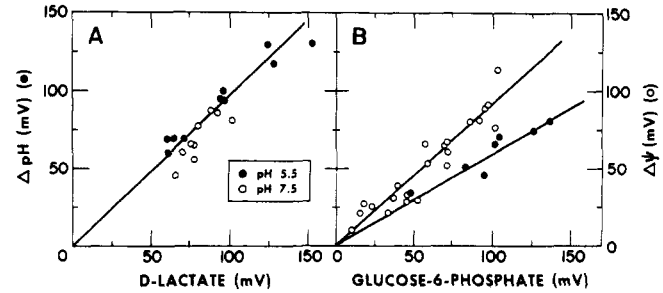

FIGURE 3: Relationship of ΔpH at pH 5.5 and ΔΨ at pH 7.5 to steady-state levels of accumulation of D-lactate (panel A) and glucose-6-P (panel B). Membrane vesicles prepared from *E. coli* ML 308-225 grown on succinate (A) and GN-2 grown on glucose-6-P (B) were transferred into 0.1 M potassium phosphate at pH 5.5 (●—●) and pH 7.5 (○—○) as described in Methods. Aliquots (0.4 mL containing about 6 mg of membrane protein) were diluted to a final volume of 0.8 mL in the upper chamber of a flow dialysis apparatus. All reaction mixtures contained (in final concentrations) 0.05 M potassium phosphate (pH 5.5 or 7.5, as indicated), 0.01 M magnesium sulfate, 12 μM $TPMP^+$, 37.5 μM sodium acetate (or 200 μM DMO), and 200 μM sodium D-lactate (A) or 70 μM disodium glucose-6-P (B). In some experiments, valinomycin (in concentrations ranging up to 5 μM), nigericin (in concentrations ranging up to 0.1 μM), or CCCP (in concentrations ranging up to 10 μM) was also added to the reaction mixture in the upper chamber, and in other experiments, valinomycin (5 μM) plus nigericin (in concentrations ranging up to 0.1 μM) were added. Under all conditions, flow dialysis was performed as described (Ramos et al., 1976; Ramos and Kaback, 1977a,b; Tokuda and Kaback, 1977) using sodium ascorbate (20 mM, final concentration) and PMS (0.1 mM, final concentration) as electron donor. For measurements of ΔΨ, [^{3}H]$TPMP^+$ (4.36 Ci/mmol) was used in place of unlabeled $TPMP^+$. For measurements of ΔpH at pH 5.5, sodium [1,2-^{14}C]acetate (54 mCi/mmol) or [2-^{14}C]DMO (11 mCi/mmol) was used in place of unlabeled sodium acetate or DMO. For measurements of steady-state levels of D-lactate (A) and glucose-6-P (B) accumulation, sodium [1-^{14}C]-D-lactate (9.1 mCi/mmol) and disodium [U-^{14}C]glucose-6-P (38.7 mCi/mmol) were used in place of unlabeled D-lactate and glucose-6-P. It should be emphasized that each experimental point represents the results of flow dialysis determinations in which ΔpH, ΔΨ, and D-lactate or glucose-6-P accumulations were measured under identical conditions.

one at pH 5.5 and a slope of 0.5 at pH 7.5, with an intermediate value for proline at pH 6.6. In other words, at pH 5.5, accumulation of lactose and proline at each value of $\Delta\bar{\mu}_{H^+}$ is equal to or slightly less than $\Delta\bar{\mu}_{H^+}$, indicating that the stoichiometry between protons and lactose or proline is 1:1 at this external pH. On the other hand, at pH 7.5, for each value of $\Delta\bar{\mu}_{H^+}$, there is approximately twice as much lactose and proline accumulation, indicating that the stoichiometry between protons and solute is 2:1 at this pH.

Utilizing this experimental approach, it is possible to "titrate" the increase in proton:proline stoichiometry as a function of pH. Each experimental point shown in Figure 2 represents a series of flow dialysis determinations in which the steady-state level of proline accumulation was plotted against $\Delta\bar{\mu}_{H^+}$ over a wide range of values at each pH given. The data were treated as shown in Figure 1, and the apparent proton:proline stoichiometries were calculated at each pH (i.e., the inverse slope of each function) and plotted as a function of external pH. It is apparent that at pH 5.0 and 5.5, the stoichiometry values approximate unity. Subsequently, as external pH increases from pH 5.5 to pH 7.5, the values increase progressively to a stoichiometry of two at pH 7.5 and 8.0, with a midpoint at about pH 6.8. Thus, it can be stated that the apparent p*K* of the functional group responsible for the increase in proton:proline stoichiometry is approximately 6.8.

As opposed to lactose and proline, accumulation of D-lactate and glucose-6-P, among other organic acids, is coupled preferentially to ΔpH at pH 5.5 (Ramos and Kaback, 1977b). Importantly, moreover, the transport of these solutes is coupled to ΔΨ at pH 7.5 where there is no ΔpH across the membrane (Padan et al., 1976; Ramos et al., 1976; Ramos and Kaback, 1977a,b). These observations and others (Ramos and Kaback, 1977b) have led to the suggestion that transport of these organic acids occurs by an electrically neutral mechanism at pH 5.5 as postulated by Mitchell (1973; Harold, 1972), but at pH 7.5, by an electrogenic symport mechanism in which an additional proton(s) is translocated in association with the carriers. The data presented in Figure 3 provide direct support for this notion. In the experiments shown, ΔpH was measured at pH 5.5 under various conditions and compared with the steady-state levels of D-lactate (Figure 3A) and glucose-6-P (Figure 3B) accumulation measured under the same conditions. Accumulation of both organic acids at pH 5.5 varies directly with ΔpH over the range of values tested, indicating that the transport mechanisms are electrically neutral at this pH. It is also apparent that the relationship between D-lactate accumulation and ΔpH exhibits a slope of one, an observation which is consistent with a proton:D-lactate stoichiometry of 1:1. With glucose-6-P, on the other hand, the slope of the function is clearly less than unity and approximates a value of 0.6, indicating that more than one proton is taken up with the sugar-P at this pH. Since the phosphate moiety in glucose-6-P has two p*K*s (pK_1 = 0.94; pK_2 = 6.11), this finding is not surprising and, in fact, provides convincing support for the validity of the experimental methods utilized.[2] In any event, when D-lactate and glucose-6-P transport are measured at pH 7.5 under a variety of conditions and related to ΔΨ, it is evident that accumulation of both organic acids bears a linear relationship to ΔΨ and that both functions exhibit slopes of one. Thus, accumulation of D-lactate and glucose-6-P becomes electro-

[2] The stoichiometry between protons and glucose-6-P exhibits a value of less than two because the pH of the experiments is below the second p*K* of the phosphate moiety of glucose-6-P (i.e., pK_2 = 6.11). However, using the following equation and eq 4 with experimentally determined values for the glucose-6-P concentration gradient (i.e., 100) and external pH of 6.0 (Figure 3), the theoretical stoichiometry (i.e., *z*, see Discussion) can be calculated:

$$\frac{A_{T_{in}}}{A_{T_{out}}} = \frac{[(H^+)_{in}^2 + K_{a_1}(H^+)_{in} + K_{a_1}K_{a_2}](H^+)_{out}^2}{[(H^+)_{out}^2 + K_{a_1}(H^+)_{out} + K_{a_1}K_{a_2}](H^+)_{in}^2}$$

where $(H^+)_{out} = 10^{-6}$, $K_{a_1} = 0.115$, $K_{a_2} = 7.7 \times 10^{-7}$, and $A_{T_{in}}/A_{T_{out}} = 100$. Solving for $(H^+)_{in}$ yields a value of 6.8×10^{-8} (i.e., $pH_{in} = 7.16$). Thus, ΔpH = 1.16. Substituting this value for ΔpH and a value of 100 for the glucose-6-P concentration gradient into eq 4, a value of 1.72 is calculated for *z*. Essentially the same value is obtained with other experimental points shown in Figure 3. Clearly, this value is in excellent agreement with the experimentally determined stoichiometry of approximately 1.7.

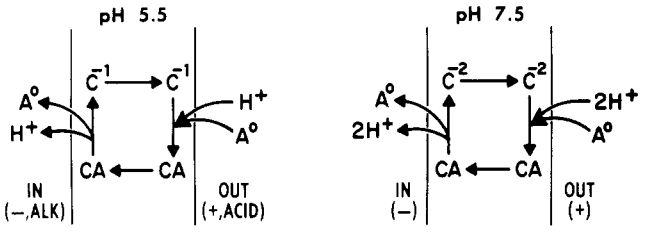

FIGURE 4: Model for symport of neutral substrates at pH 5.5 and pH 7.5. C represents a macromolecular carrier or porter; A represents the substrate; and the superscripts represent charge.

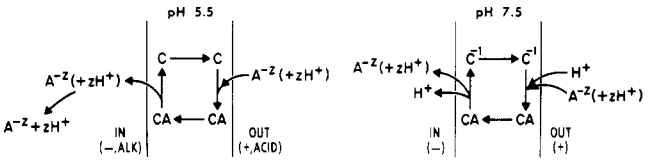

FIGURE 5: Model for transport of certain anionic substrates at pH 5.5 and pH 7.5. C represents a macromolecular carrier or porter; $A^{-z}(+zH^+)$ represents a substrate of $-z$ charge with z protons bound; and $A^{-z} + zH^+$ represents a substrate of $-z$ charge in dissociated form.

genic at pH 7.5 by virtue of the symport of one additional proton.

Discussion

The experiments presented in this paper provide strong support for the contention that the stoichiometry between protons and substrate during respiration-linked active transport may increase as a function of pH. As such, the results represent an important extension of the chemiosmotic hypothesis (Mitchell, 1961, 1966, 1968, 1973; Harold, 1972) with respect to active transport. It should be noted, however, that Mitchell (1968) has mentioned the theoretical implication of proton: substrate stoichiometries in excess of unity.

The experimental results presented here are best explained within the framework outlined by Rottenberg (1976) in which certain well-defined models were presented. Accordingly, it is assumed that the ternary complex between a carrier, protons, and substrate is always neutral during translocation and that the net charge of the complex is determined by the net charge of the substrate, the number of protons that are translocated with the substrate, and the charge on the carrier which may be either negative or neutral. The overall mechanism of the transport cycle occurs in the following manner (Figures 4 and 5): (i) binding of protons and substrate to the carrier at the external surface of the membrane; (ii) equilibration of the uncharged ternary complex between the outer and inner surfaces of the membrane; (iii) dissociation of protons and substrate from the inner surface of the membrane; and (iv) return of the unloaded carrier to the outer surface of the membrane, a process which would be facilitated by the membrane potential (interior negative) if the carrier is negatively charged (Schuldiner et al., 1975; Rudnick et al., 1975, 1976).

With lactose and proline which are neutral substrates, the charge on the unloaded carrier must be negative and the valency of the ternary complex equal to the number of protons translocated in symport with the substrate in order to conform to the postulate that the ternary complex is neutral (Figure 4). If the symport system is completely coupled, it is driven by the sum of the electrochemical potential difference of the transported species and would approach a steady-state when:

$$\Delta\bar{\mu}_A + n\Delta\bar{\mu}_{H^+} = 0 \qquad (2)$$

where $\Delta\bar{\mu}_A$ is the electrochemical potential of the substrate, $\Delta\bar{\mu}_{H^+}$ is the proton electrochemical potential, and n is the number of protons translocated in symport with the substrate (i.e., the stoichiometry of the reaction). By combining eq 1 and 2, the following relationship is obtained for neutral substrates (cf. Rottenberg (1976) for the details of this and subsequent derivations):

$$Z \log(A^0_{in}/A^0_{out}) = -n(\Delta\Psi - Z\Delta pH) = -n\Delta\bar{\mu}_{H^+} \qquad (3)$$

where $Z = 2.3RT/F$ and A^0 denotes a neutral substrate. Thus, for $n = 1$

$$Z \log(A^0_{in}/A^0_{out}) = -\Delta\bar{\mu}_{H^+}$$

and for $n = 2$

$$Z \log(A^0_{in}/A^0_{out}) = -2\Delta\bar{\mu}_{H^+}$$

Clearly, the experimental results presented in Figure 1 are in complete agreement with these considerations (Figure 4). At pH 5.5, where $\Delta\bar{\mu}_{H^+}$ is relatively high, the steady-state levels of accumulation of both lactose and proline appear to be essentially in equilibrium with $\Delta\bar{\mu}_{H^+}$ over a range of values, and the stoichiometry is equal to unity (i.e., $n = 1$). At pH 7.5 on the other hand, $\Delta\bar{\mu}_{H^+}$ is relatively low, and the data are consistent with a stoichiometry of two (i.e., $n = 2$). Moreover, evidence has been presented (Figure 2) that the functional group in the proline carrier which is responsible for the change in stoichiometry has a pK of about 6.8.

As opposed to the neutral substrates, D-lactate and glucose-6-P are anions, and their respective carriers need not be negatively charged in order to maintain electroneutrality since balancing of charge can be accomplished by binding of protons to the anionic substrates themselves (Figure 5). When transport of these anions approaches a steady-state, eq 2 determines the distribution of substrate; however, in this case, by combining eq 1 and 2, the following relationship is obtained for a neutral carrier with a substrate of charge $-z$ (Rottenberg, 1976):

$$\log(A^{-z}_{in}/A^{-z}_{out}) = z\Delta pH \qquad (4)$$

Obviously, this relationship can account for the data obtained with D-lactate and glucose-6-P at pH 5.5 (Figure 3). Accumulation of both substrates depends primarily on ΔpH at this external pH (in addition, see Ramos and Kaback, 1977b), and D-lactate, which has one pK, exhibits a stoichiometry of one, while glucose-6-P, which has two pKs, exhibits a stoichiometry of about 1.7.[2]

If the same carrier becomes negatively charged at higher pH (Figure 5), $n = z + 1$ and

$$Z \log(A^{-z}_{in}/A^{-z}_{out}) = \Delta\Psi + nZ\Delta pH = -\Delta\bar{\mu}_{H^+} + zZ\Delta pH \qquad (5)$$

At pH 7.5, however, there is no ΔpH across the vesicle membrane (Ramos et al., 1976; Ramos and Kaback, 1977a,b), and accumulation should be in equilibrium with $\Delta\Psi$, a prediction which is fulfilled by the data presented in Figure 3 (in addition, see Ramos and Kaback, 1977b).

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5-Iodoacetamidofluorescein-Labeled Chloroplast Coupling Factor 1: Conformational Dynamics and Labeling-Site Characterization†

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ABSTRACT: Physical and spectroscopic properties of 5-iodoacetamidofluorescein (5-IAF), a new sulfhydryl-specific fluorescent label, are described. Under certain conditions, 5-IAF labels the chloroplast phosphorylation coupling factor (CF) predominantly on the β subunit. Approximately 88% of the ATPase activity and over 60% of the ability of CF to reconstitute photophosphorylation are retained after labeling. Trypsin activation of the ATPase activity of 5-IAF-labeled CF dramatically alters the fluorescence properties at the labeling site, indicating its involvement in ATPase activation. Studies of the fluorescence emission spectra, fluorescence polarization, and potassium iodide quenching of 5-IAF and 5-IAF-labeled CF demonstrate that the labeling site is in a partially buried hydrophobic region which is partially accessible to potassium iodide quenching from the solvent phase and which restricts the motion of the fluorescent label. The fluorescence shows little change upon substrate binding. We conclude that the label is located in a cleft region remote from the enzyme active site. Variation of the reaction pH between 6.4 and 8.5 significantly alters the number of attached labels. ATP decreases the extent of labeling over the entire pH range studied. These labeling changes reflect substrate- and pH-induced conformational changes in CF. Certain interdependences observed in these conformational changes suggest that the transmembrane electrochemical gradient may directly induce conformational changes in CF leading to net ATP synthesis. The γ subunit plays a central role in the expression of these intersubunit conformational changes.

The chloroplast coupling factor (CF[1]) is a water-soluble protein associated with the thyalkoid membrane surface, where it catalyzes the terminal step of ATP production during photophosphorylation. It contains five different subunit types ranging in size from 13 000 to 59 000 daltons (Nelson et al., 1973). Two tight-binding sites for ATP or ADP have been characterized (Livne and Racker, 1969; Roy and Moudrianakis, 1971; Cantley and Hammes, 1975a) along with other, weaker sites. These sites have been tentatively assigned to the α and β subunits (Cantley and Hammes, 1975a). This assignment is verified by the observation that an α and β subunit complex derived from CF is active as an ATPase (Deters et al., 1975).

In a previous investigation, covalent labeling of tyrosine residues on the β subunit with NBD-Cl led to 80% inhibition of the enzyme activity (Deters et al., 1975). We have used a new sulfhydryl-specific fluorescent label, 5-iodoacetamidofluorescein (5-IAF), to label CF with minimal decrease in the enzyme activity. The label reacts preferentially with the β subunit at pH 7.8 in the presence of ATP. Investigation of the fluorescence emission spectrum, the fluorescence polar-

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[1] Abbreviations used are: CF, chloroplast coupling factor 1; 5-IAF, 5-iodoacetamidofluorescein; TLC, thin-layer chromatography; NEM, *N*-ethylmaleimide; DCCD, *N,N'*-dicyclohexylcarbodiimide; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; Chl, chlorophyll; EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate.