Effect of Extracellular pH on the Respiratory Chain and Energetics of *Gluconobacter suboxydans*[†]

Kazunobu MATSUSHITA, You-ichiro NAGATANI, Emiko SHINAGAWA,
Osao ADACHI and Minoru AMEYAMA

Laboratory of Applied Microbiology, Department of Agricultural Chemistry,
Faculty of Agriculture, Yamaguchi University,
Yamaguchi 753, Japan

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Gluconobacter suboxydans contains a large amount of cytochrome *c* in addition of cytochrome *o* in the respiratory chain of the membrane. The content of cytochrome *c* was found to be influenced by the extracellular pH in growth which is lowered by oxidation products of several sugars or sugar alcohols in the growth medium. Lowering of the external pH caused an increase in cytochrome *c* content concomitantly with an increase in alcohol dehydrogenase activity, while neither cytochrome *o* content nor glucose dehydrogenase activity is increased. The increase in cytochrome *c* content was also accompanied by an increase in KCN-insensitivity for the respiratory activity. The change in cytochrome *c* content appeared to be mainly due to the change of a cytochrome *c* corresponding to the second subunit of alcohol dehydrogenase. Furthermore, the extracellular pH of *G. suboxydans* also affected a respiration-driven proton translocation; the H^+/O ratio of the cells grown at lower pH was much lower than the value, about 2, of the cells grown at higher pH. In the presence of KCN, however, both the cells grown at high and low pHs could not generate a membrane potential. Thus, this study indicates that *G. suboxydans* produces a KCN-insensitive and non-energy-generating respiratory bypass, which may be related to a cytochrome *c* associated with alcohol dehydrogenase, concomitant with a decrease of the extracellular pH during growth.

Gluconobacter strains are well known to be oxidative bacteria which not only oxidize ethanol to acetic acid and D-glucose to D-gluconic acid, but which are also ketogenic on sugars such as D-gluconic acid, D-sorbitol, glycerol and so on.¹⁾ These sugar oxidation reactions are performed with each respective primary dehydrogenase linked to the electron transport system located in the cytoplasmic membrane of the organism.²⁾

We have already characterized several primary dehydrogenases involved in the sugar oxidation reaction. It has been shown that glucose, alcohol, aldehyde, and glycerol dehydrogenases are quinoproteins containing pyrroloquinoline quinone as the prosthetic group^{3,4)} while gluconate, 2-ketogluconate, and sorbitol dehydrogenases are flavoprotein

containing covalently-bound flavin as the prosthetic group.^{5,6)} Furthermore, the sugar-oxidizing respiratory chain has been characterized in *G. suboxydans*, where cytochromes *c* and *o* are present in the respiratory chain which branches with KCN-sensitive and insensitive terminal oxidases.⁷⁾ The KCN-sensitive terminal oxidase has been purified and characterized as an energy-generating cytochrome *o* oxidase.⁸⁾

In this study, we show that the extracellular pH of the cells regulates one of the two terminal oxidase systems, a KCN-insensitive and non-energy-generating bypass, which is also reported to be related to a cytochrome *c* associated with alcohol dehydrogenase.

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Materials and Methods

Materials. Ubiquinone-2 (Q_2) was kindly supplied by Eizai Co., and Q_2H_2 was prepared by the method of Rieske.⁹⁾ 3,3'-Diisopropylthiodicarbocyanine [$diS-C_3(5)$] and bis (1,3-diethylbarbituric acid) pentamethine oxonol [$diBA-C_2(5)$] were obtained from Molecular Probes. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was from Sigma.

Bacterial strain and growth conditions. *G. suboxydans* IFO 12528 was grown up to the late logarithmic phase in sugar-rich medium,⁷⁾ the pH of which was controlled by adding 50 mM potassium phosphate (K phosphate).

Preparation of membranes and inside-out vesicles. Cells were harvested by centrifugation, and then washed twice with distilled water. For preparation of membranes, washed cells were resuspended in 10 mM K phosphate (pH 6.0), and passed through a French pressure cell press at 16,000 psi. The membranes were prepared from the supernatant, as described previously,⁷⁾ and resuspended in 50 mM K phosphate (pH 6.5). To prepare inside-out vesicles, washed cells were resuspended in 50 mM K phosphate (pH 6.5) containing 5 mM $MgSO_4$ and then the vesicles were prepared by French press at low shear forces.¹⁰⁾ The vesicles were resuspended in the same buffer for the following experiments.

Preparation of right-side-out vesicles. Cell were converted into spheroplasts with lysozyme in two different buffer systems, 0.1 M Tris (pH 8.0) containing 20% sucrose and 1 mM EDTA or 0.1 M Tris (pH 8.0) containing 0.5 M NaCl and 1 mM EDTA, as described previously.¹¹⁾ The former buffer system was used for the cells grown at high pH, and the latter system for the cells grown at low pH. The spheroplasts thus prepared were suspended in 50 mM K phosphate (pH 6.5) containing 5 mM $MgSO_4$ at a concentration of 0.17 g wet cells per ml. The suspension was passed through a French press at 5,000 psi (low pressure) after DNase was added to 20 μ g per ml. After centrifugation at $2,000 \times g$ for 10 min to remove the unbroken spheroplasts and the debris, the supernatant was centrifuged at $46,000 \times g$ for 30 min. The precipitate was suspended in 50 mM K phosphate (pH 6.5) for the following experiments.

Enzyme assays. Oxidase activity for ethanol or glucose was measured polarographically with a Clark-type oxygen electrode.⁷⁾ Alcohol or glucose dehydrogenase activity was measured spectrophotometrically with phenazine methosulfate and dichlorophenol indophenol as electron acceptors.¹²⁾ Q_2H_2 oxidase activity was measured spectrophotometrically at 25°C by following the increase in absorbance at 275 nm. The reaction mixture contained 50 mM K phosphate (pH 6.5), 30 μ M Q_2H_2 , 0.02% Tween

20, and membranes in a total volume of 1 ml. The activity was calculated using a millimolar extinction coefficient of 12.25.

Measurement of respiratory-driven proton translocation. Changes in external pH in intact cells were measured with a Radiometer pH meter based on an oxygen pulse method, as described previously.¹⁴⁾ For the experiment, cells were harvested by centrifugation at $6,200 \times g$ for 10 min, washed twice with 150 mM KCl, and then suspended with the same solution at a concentration of 0.05 g (wet weight) per ml. The cell suspensions were aerated for 2–3 hr by rotary shaking at 30°C. The reaction mixture contained 100 mM KSCN, 10 mM electron donor, and 1 ml of the cell suspension in a total volume of 3 ml (final KCl concentration of 50 mM). The mixture was adjusted initially to pH 6.0–6.5 with 0.1 N KOH or HCl and then continuously flushed with water-saturated argon. The reaction was started by adding 20 μ l of 150 mM KCl and calibrated by the addition of 10 μ l of deoxygenated 1 mM HCl.

Measurement of membrane potential. Generation of a membrane potential (inside negative) in right-side-out vesicles was measured by monitoring the fluorescence quenching of $diS-C_3(5)$.¹³⁾ The reaction mixture (1 ml total volume) contained 50 mM K phosphate (pH 6.5), 5 mM $MgSO_4$, 1 μ M $diS-C_3(5)$, 10 mM electron donor, and vesicles. The membrane potential (inside positive) in inside-out vesicles was monitored by following the fluorescence quenching of $diBA-C_2(5)$.¹⁰⁾ The reaction mixture (1 ml total volume) contained 50 mM K phosphate (pH 6.5), 5 mM $MgSO_4$, 32 μ M $diBA-C_2(5)$, 10 mM electron donor, and vesicles.

Other analytical procedures. Cytochromes *c* and *o* contents were measured from a reduced minus oxidized and a carbon monoxide-reduced minus reduced difference spectra, respectively, as described.^{7,8)} The low temperature-difference spectrum was taken in liquid nitrogen in a cuvette of 2 mm light path. Heme-staining of sodium dodecyl sulfate-polyacrylamide gel was performed with heme-catalyzed peroxidase activity.¹⁵⁾ Protein content was determined by a modification of Lowry's method.¹⁶⁾

Results

Effect of extracellular pH in growth on membrane-bound cytochromes of G. suboxydans

When *G. suboxydans* was grown at different pHs that were controlled by adding 50 mM K phosphate to the growth medium, the cytochrome *c* content in the membranes increased as the external pH decreased, while the cytochrome *o* content remained unchanged (Fig.

Fig. 1. Effect of Extracellular pH on Cytochromes *c* and *o* Contents in the Membranes of *G. suboxydans*.

The contents of cytochrome *c* (*cyt c*) and cytochrome *o* (*cyt o*) were measured by the method described in Materials and Methods. Growth pH shows the final pH in the growth medium.

1). As can be seen in Fig. 2, low temperature reduced minus oxidized difference spectra of the membranes prepared from the cells grown under high pH clearly show a shoulder at 563 nm, deriving from cytochrome *o*, which is not obvious due to a large amount of cytochrome *c* in the membranes from the cells grown at low pH. Using the supernatant solubilized from the membranes with 1% Triton X-100 that has been known to contain almost all the cytochromes *c*,⁷⁾ species of cytochrome *c* were examined by heme-staining in sodium dodecyl sulfate gel electrophoresis. As shown in Fig. 3, the solubilized supernatant contains two major heme-staining bands, of which molecular weights were about 80,000 and 50,000. As shown, it is obvious that the second hemoprotein band, 50 kdaltons, is largely increased in the membranes prepared from the cells grown at acidic pH while the first hemoprotein band, 80 kdaltons, is increased a little.

Effect of extracellular pH on respiratory enzyme activities of G. suboxydans

Several respiratory activities were also examined in relation to extracellular pH dur-

Fig. 2. Low Temperature-difference Spectra of the Membranes from *G. suboxydans* Grown at High and Low pHs.

Membranes were prepared from the cells grown at final pH 4.5 (A, protein concentration of 10.7 mg/ml) and at pH 6.4 (B, protein concentration of 10.7 mg/ml).

Fig. 3. Heme Staining Patterns in Sodium Dodecyl Sulfate-polyacrylamide Gel with Triton X-100-solubilized Fractions of the Membranes Prepared from *G. suboxydans* Grown at High and Low pHs.

Membranes were prepared from the cells grown at final pH 4.4 (pH 4) and at pH 6.2 (pH 6), and then extracted with 1% Triton X-100 at a protein concentration of 10.0 mg/ml. The solubilized supernatants (each 28 μ g) were treated with 3% sodium dodecyl sulfate and then put on a 12.5% gel. Heme staining was done as described in Materials and Methods, and densitometric patterns were obtained. Hemoproteins were observed at molecular weights of about 80,000 (I) and 50,000 (II).

ing the growth of the organism. As shown in Fig. 4, alcohol dehydrogenase activity is largely increased, though variably, by decreases of the pH in the growth medium, while glucose dehydrogenase activity is not changed by the external pH. The fluctuation of the alcohol dehydrogenase activity at the low external pH

Fig. 4. Effect of Extracellular pH on Several Enzyme Activities of *G. suboxydans* Respiratory Chain.

Each enzyme activity was measured as described in Materials and Methods. Alcohol (●) and glucose (○) dehydrogenases.

may be due to apo-enzyme formation, which has recently been shown to occur at acidic pH in growth of *G. suboxydans*.¹⁷⁾ External pH during growth also affected the KCN-sensitivity of the respiratory chain. As shown in Fig. 5, either ethanol, glucose, or NADH oxidase activity in the membranes prepared from the cells grown at pH 6.3 is more sensitive to KCN than grown at pH 4.3.

*Effect of extracellular pH on respiratory-driven proton translocation in *G. suboxydans* cells*

The H^+/O ratio was measured in the cells grown at high and low pHs. A rapid and transient acidification of the external medium was observed by the addition of oxygen into the anaerobic cell suspension (Fig. 6). As shown, the acidification is significantly higher in the cells grown at higher pH (6.1) than in the cells grown at lower pH (4.3). However, the acidification did not return completely to the initial pH baseline, and this small but stable pH change was observed even in the presence of an uncoupler, CCCP, which suggests there is an acid produced by the oxidation of substrate, e.g., acetic acid from ethanol. Thus, the H^+/O ratio was calculated from the difference between the extrapolated initial pH change and the pH change obtained in the presence of CCCP. As summarized in Table I, the H^+/O ratio in the cells grown at high pH approximates 2 with any respiratory substrates,

Fig. 5. Effect of KCN on Several Oxidase Activities in the Membranes from *G. suboxydans* Grown at High and Low pHs.

Membranes were prepared from the cells grown at final pH 6.3 (●—●) and at pH 4.3 (○—○). Ethanol, glucose and NADH oxidase activities were measured as described in Materials and Methods.

while the value of the cells grown at low pH is much lower than 2.

Effect of extracellular pH on membrane potential generation in membrane vesicles of G. suboxydans

Membrane potential generation was measured fluorometrically using carbocyanine in right-side-out vesicles and also using oxonol in inside-out vesicles. As shown in Fig. 7, in the right-side-out vesicles, a fluorescence quenching of diS-C₃(5) is induced by adding respiratory substrate, which is enhanced with nigericin and diminished with valinomycin. Nigericin is known to collapse pH gradient

Table 1. H⁺/O RATIO MEASURED IN *G. suboxydans* CELLS GROWN AT HIGH AND LOW pHs

Respiratory substrates	H ⁺ /O Ratio ^a	
	Cells grown at pH 4	Cells grown at pH 6
Endogenous	1.5 ± 0.2 (4)	2.3 ± 0.4 (3)
Ethanol	1.2 ± 0.2 (7)	2.2 ± 0.2 (3)
Glucose	1.1 ± 0.2 (5)	1.8 ± 0.3 (3)
Glycerol	1.3 ± 0.1 (4)	1.7 ± 0.4 (3)
Lactate	1.2 ± 0.2 (5)	1.8 ± 0.4 (3)

^a Data show the mean value and the standard deviations from several experiments. Parentheses show the number of the experiments.

with an increase of membrane potential, and valinomycin to collapse membrane potential, which suggest that the membrane vesicles of *G. suboxydans* generate an electrochemical proton gradient during oxidation of the respiratory substrate.

Fig. 6. External pH Changes Following Oxygen Supply to Anaerobic Cell Suspensions of *G. suboxydans* Grown under High and Low pHs.

Cells were grown at final pH 4.3 (pH 4) and at pH 6.1 (pH 6), and pH changes were measured as described in Materials and Methods. In the absence or presence of 10 μM CCCP, reactions were started by addition of 20 μl of air-saturated 150 mM KCl (9.96 natom of O₂) and 10 nmol of argon-flushed HCl was introduced at the end of the reaction for calibration.

Fig. 7. Fluorometric Trace of Membrane Potential Generation in Right-side-out Vesicles from *G. suboxydans* Grown at Low pH.

Generation of membrane potential (inside negative) was monitored by fluorescence quenching of diS-C₃(5) as described in Materials and Methods. (A) Reaction was started by the addition of 10 mM ethanol (Et), followed by the addition of 25 nM nigericin (Nig), 1 mM KCN, and then 1 μM valinomycin (Val). (B) On the way of the reaction, 1 mM KCN was added and then aeration (O₂) was done by shaking at the end. (C) 1 μM Val was added at the end of the reaction. The reaction mixture contained right-side-out vesicles (0.05 mg of protein) prepared from the cells grown at final pH 4.3.

Fig. 8. Fluorometric Trace of Membrane Potential Generation in Inside-out Vesicles from *G. suboxydans* Grown at High pH.

Generation of membrane potential (inside positive) was monitored by fluorescence quenching of diBA-C₂ (5) as described in Materials and Methods. (A) Reaction was started by the addition of 10 mM ethanol (Et), followed by the addition of 25 nM Nig, and then the reaction ceased after full consumption of oxygen resolved in the reaction mixture. (B) 0.03 mM KCN was added during the reaction, and then oxygen was introduced again by shaking after the reaction ceased for the consumption of oxygen. (C) 1 mM KCN was added during the reaction, followed by aeration and by the addition of 1 μ M Val. The reaction mixture contained inside-out vesicles (0.17 mg of protein) prepared from the cells grown at final pH 5.9.

The fluorescence quenching of carbocyanine in the right-side-out vesicles was inhibited by the addition of 1 mM KCN, and the inhibition was not much enhanced with further addition of valinomycin (Fig. 7A) and not restored by the addition of oxygen (Fig. 7B), suggesting that 1 mM KCN inhibits the generation of a membrane potential almost completely. As summarized in Table II, the intensity of membrane potential generated is almost the same, though not quantitative, between the right-side-out vesicles of the cells grown at high and low pHs, and KCN (1 mM) diminishes the membrane potential almost completely in the membrane vesicles of both cells.

Membrane potential generation was also observed in the inside-out vesicles of *G. suboxydans* (Fig. 8). The addition of ethanol caused a fluorescence quenching of diBA-C₂ (5), which was stimulated with nigericin and returned to the baseline after full consumption of oxygen (Fig. 8A). Low concentration of KCN (0.03 mM) caused a partial de-quenching and thus re-introduction of oxygen into the reaction mixture induced a partial quenching (Fig. 8B). The addition of 1 mM KCN caused a complete de-quenching which is not restored

Table II. MEMBRANE POTENTIALS (INSIDE NEGATIVE) GENERATED IN RIGHT-SIDE-OUT VESICLES PREPARED FROM *G. suboxydans* GROWN AT HIGH AND LOW pHs

Respiratory substrates	Cells grown at pH 4		Cells grown at pH 6	
	$\Delta F/F$ (%)	Inhibition with KCN (%)	$\Delta F/F$ (%)	Inhibition with KCN (%)
Ethanol	58	91	59	90
Glucose	55	88	67	92

Membrane potential (inside negative) was measured with diS-C₃ (5). As shown in Fig. 7, 1 mM KCN and finally 1 μ M valinomycin were added after the quenching became steady. Inhibition (%) with KCN was calculated as the value of de-quenching obtained with KCN versus that with valinomycin.

totally by the addition of oxygen (Fig. 8C). Thus, the inhibition of oxonol quenching with KCN was compared between the inside-out vesicles from the cells grown at high and low pHs (Fig. 9). As shown, KCN inhibits the membrane potential generation of both vesicles with the same magnitude. Thus, it has been shown that membrane potential generation in the membrane vesicles of *G. sub-*

Fig. 9. Effect of KCN Concentration on Oxonol Quenching in the Inside-out Vesicles Prepared from *G. suboxydans* Grown at High and Low pHs.

Fluorescence quenching induced with ethanol was measured as shown in Fig. 8, and the remaining activity (%) was calculated from the quenching produced by re-addition of oxygen versus the original quenching induced with ethanol before addition of KCN (see Fig. 8B and C). (A) Inside-out vesicles prepared from the cells grown at pH 4.3 and (B) vesicles prepared from the cells grown at pH 6.1.

oxydans is KCN-sensitive regardless of the external pH during growth of the cells.

Discussion

In this study, the cytochrome *c* content in the membranes of *G. suboxydans* has been shown to change greatly depending on its extracellular pH during the growth. Lowering of the external pH increased the cytochrome *c* content as well as the activity of alcohol dehydrogenase, while the cytochrome *o* content and other enzyme activities of the respiratory chain were not affected. Furthermore, the increase in cytochrome *c* content was shown to be mainly due to the increase in a cytochrome *c* component of 50 kdaltons, which may correspond to the second subunit of alcohol dehydrogenase or cytochrome *c*₅₅₃ (CO).⁷⁾ Since, both cytochromes *c* recently have been demonstrated to be the same entity (unpublished data), the external pH during the growth seems to regulate synthesis of the cytochrome *c* component of alcohol

dehydrogenase, which may, as a result, affect the activity of alcohol dehydrogenase.

The results presented here have also indicated that KCN-sensitivity of the respiratory activity in the membranes of *G. suboxydans* is decreased by lowering of the external pH. This finding implies that the KCN-insensitive terminal oxidase may be regulated by the external pH, since the respiratory chain of *G. suboxydans* has been shown to branch at the terminal end with KCN-sensitive and insensitive terminal oxidases⁷⁾ and the content of cytochrome *o*, shown to be a KCN-sensitive terminal oxidase,⁸⁾ was not changed by the external pH. As expected from the change of the terminal oxidases, the energetics of *G. suboxydans* is also affected by the external pH. This was shown by the measurement of the H^+/O ratio in the cells, the value of which was decreased from about 2.0 to 1.2 by the external pH changing from 6.0 to 4.0. Since the KCN-sensitive terminal oxidase, cytochrome *o*, has been shown to generate an electrochemical proton gradient by the oxidation of ubiquinol,⁸⁾ the respiratory chain containing cytochrome *o* as a sole terminal oxidase is expected to yield an H^+/O ratio of 2 by analogy to *E. coli* cytochrome *o*.¹⁸⁾ Thus, an expected KCN-insensitive terminal oxidase that is produced at low external pH during the growth seems to have no coupling site, and therefore decreases the H^+/O ratio of the respiratory chain of the cells grown at low pH. This consideration is supported by the observation that no membrane vesicles of *G. suboxydans*, whichever the cells are grown at high and low pHs, are able to generate membrane potential in the presence of KCN to block KCN-sensitive cytochrome *o* oxidase.

Thus, these data indicate that the increase in cytochrome *c* component, which seems to be the second subunit of alcohol dehydrogenase, is accompanied by the increase in a KCN-insensitive and non-energy-generating terminal oxidase activity, thus leading us a tentative conclusion that the cytochrome *c* being regulated by the external pH constitutes the KCN-resistant bypass. However, there re-

mains some questions to be overcome; although the cells grown at high pH exhibit a maximum H^+/O ratio of 2.0, which is expected only in the presence of cytochrome *o* terminal oxidase, the cells still contain a minimum amount of the second subunit of alcohol dehydrogenase and show some KCN-insensitivity. In order to demonstrate the tentative conclusion, furthermore, KCN-insensitive terminal oxidase activity has to be reconstituted using the cytochrome *c* component of alcohol dehydrogenase. These are currently under investigation together with that of the physiological meaning of pH-dependent and non energy-generating respiratory bypass of *G. suboxydans*.

References

- 1) T. Asai, "Acetic Acid Bacteria," Tokyo Univ. Press, Tokyo, 1968, pp. 124~203.
- 2) K. Matsushita, M. Nonobe, E. Shinagawa, O. Adachi and M. Ameyama, *Agric. Biol. Chem.*, **49**, 3519 (1985).
- 3) M. Ameyama, K. Matsushita, Y. Ohno, E. Shinagawa and O. Adachi, *FEBS Lett.*, **130**, 179 (1981).
- 4) M. Ameyama, E. Shinagawa, K. Matsushita and O. Adachi, *Agric. Biol. Chem.*, **49**, 1001 (1985).
- 5) W. McIntire, T. P. Singer, M. Ameyama, O. Adachi, K. Matsushita and E. Shinagawa, *Biochem. J.*, **231**, 651 (1985).
- 6) E. Shinagawa, K. Matsushita, O. Adachi and M. Ameyama, *Agric. Biol. Chem.*, **46**, 135 (1982).
- 7) M. Ameyama, K. Matsushita, E. Shinagawa and O. Adachi, *Agric. Biol. Chem.*, **51**, 2943 (1987).
- 8) K. Matsushita, E. Shinagawa, O. Adachi and M. Ameyama, *Biochim. Biophys. Acta*, **894**, 304 (1987).
- 9) J. S. Rieske, *Methods in Enzymol.*, **10**, 239 (1967).
- 10) K. Matsushita, T. Ohnishi and H. R. Kaback, *Biochemistry*, **26**, 7732 (1987).
- 11) K. Matsushita, E. Shinagawa, O. Adachi and M. Ameyama, *Agric. Biol. Chem.*, **45**, 1515 (1981).
- 12) K. Matsushita, Y. Ohno, E. Shinagawa, O. Adachi and M. Ameyama, *Agric. Biol. Chem.*, **44**, 1505 (1980).
- 13) K. Matsushita, L. Patel, R. B. Gennis and H. R. Kaback, *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 4889 (1983).
- 14) K. Matsushita, M. Yamada, E. Shinagawa, O. Adachi and M. Ameyama, *J. Biochem.*, **93**, 1137 (1983).
- 15) P. E. Thomas, D. Ryan and W. Levin, *Anal. Biochem.*, **75**, 168 (1976).
- 16) J. R. Dulley and P. A. Grieve, *Anal. Biochem.*, **64**, 136 (1975).
- 17) E. Shinagawa, K. Matsushita, O. Adachi and M. Ameyama, *Agric. Biol. Chem.*, **53**, 1823 (1989).
- 18) K. Matsushita, L. Patel and H. R. Kaback, *Biochemistry*, **23**, 4703 (1984).