A Reaction-induced Fourier Transform-Infrared Spectroscopic Study of the Lactose Permease

A TRANSMEMBRANE POTENTIAL PERTURBS CARBOXYLIC ACID RESIDUES

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In chemiosmotic coupling, a transmembrane ion gradient is used as the source of energy to drive reactions. This process occurs in all cells, but the microscopic mechanism is not understood. Here, Escherichia coli lactose permease was used in a novel spectroscopic method to investigate the mechanism of chemiosmotic coupling in secondary active transporters. To provide a light-triggered electrochemical gradient, bacteriorhodopsin was co-reconstituted with the permease, and reaction-induced Fourier transform-infrared spectra were obtained from the co-reconstituted samples. The bacteriorhodopsin contributions were subtracted from these data to give spectra reflecting permease conformational changes that are induced by an electrochemical gradient. Positive bands in the 1765–1730 cm⁻¹ region are attributable to carboxylic acid residues in the permease and are consistent with changes of pKₐ, protonation state, or environment. This is the first direct information concerning gradient-induced structural changes in the permease at the single amino acid level. Ultimately, these structural changes facilitate galactose binding and may be involved in the storage of free energy.

In secondary active transport, proteins employ a transmembrane electrochemical potential to translocate solutes across the membrane. This process plays a central metabolic role in all living cells. The molecular mechanism by which a transporter transduces a protonmotive force into a solute gradient is the focus of our study. The lactose permease is a transmembrane protein that is responsible for the uptake of lactose into the bacterial cytoplasm (1). The permease is a member of the major facilitator superfamily; members of the major facilitator superfamly catalyze uniport, symport, or antiport (reviewed in Ref. 2). The lactose permease is a symporter that can harness the free energy stored in a proton electrochemical gradient to drive the accumulation of lactose against a concentration gradient (1). The permease transports protons and lactose with a one-to-one stoichiometry. A number of mutagenesis studies have led to models suggesting that a few ionizable residues along transmembrane segments play key roles in the mechanism of H⁺/lactose symport (3, 4). However, direct evidence regarding the mechanism of chemiosmotic coupling in the lactose permease and related transporters has not been available.

The goal of this study was to obtain dynamic information about structural changes in the permease associated with the imposition of an electrochemical gradient. To carry out this experiment, we have employed co-reconstitution (5–7) of the permease with a light-driven proton pump, bacteriorhodopsin, and reaction-induced FT-IR spectroscopy (8). Reaction-induced FT-IR spectroscopy requires that a single sample be modulated between two structural states of interest. Co-reconstituted samples provide such a light-activatable and reversible experimental system. When a laser flash is given, bacteriorhodopsin pumps protons across the membrane and creates a H⁺ electrochemical gradient (9). In this initial study, we focus on structural changes in the absence of galactose. In the absence of lactose, the protein cannot cycle through the multiple conformational states required for the transport of a H⁺ with lactose (10). However, the electrochemical gradient is expected to cause structural changes in the permease; these structural changes have been shown previously to decrease the Kₘ value for lactose by 2 orders of magnitude (11). This structural perturbation may consist of a number of possible events, including changes in protonation, hydrogen bonding, and pKₐ values of amino acid side chains, secondary and tertiary structural changes, and helix rearrangements. Vibrational spectroscopy can monitor and identify all of these changes in protein structure. Here, we report a study that is the first step in elucidating the mechanism by which a symporter responds to the imposition of an electrochemical gradient.

EXPERIMENTAL PROCEDURES

Purification and Reconstitution of Lactose Permease—Lactose permease was purified using a histidine-tagged version of the protein and methods previously described (12). Solubilized Escherichia coli membrane protein (MP) fractions that did not bind to the nickel-affinity column were retained to use as a control. Purified lactose permease was mixed with sonicated, acetone/ether washed E. coli total lipids and was reconstituted by dialysis (12). The lipid to permease molar ratio was 520:1. The same procedure was employed for the reconstitution of the MP control.

Purification of Purple Membranes Containing Bacteriorhodopsin—Halobacterium halobium growth and purple membrane isolation were performed as described previously (13). Crude purple membranes were released from cells following lysis in water containing DNase. The preparation was significantly enriched in bacteriorhodopsin by using a series of water washes and high speed centrifugations. Final purification of purple membranes was achieved by centrifugation through a 5–40% sucrose gradient at 100,000 × g for 18 h. The purple band was removed from the gradient and was washed to remove sucrose. The

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‡ The abbreviations used are: FT-IR spectroscopy, Fourier transform-infrared spectroscopy; μE, microeinstein; MP, membrane protein; bR, bacteriorhodopsin; LP, lactose permease.
same procedure was conducted with reconstituted MP and bR, except that the permease was omitted. As a third control (bR), purple membrane was mixed with samples of sonicated, acetonite/ether washed E. coli lipids, which had been subjected to the same procedure except that the permease was omitted. As a third control (bR + MP), the same procedure was conducted with reconstituted MP and bR.

**Activity Assays**—The activity of lactose permease was measured using a counterflow assay at room temperature (12). Proteoliposomes were incubated in a pH 6.0 phosphate buffer (100 mM phosphate, pH 6.0, and 2.0 mM MgSO4) or a pH 7.4 HEPES buffer (10 mM HEPES-NaOH containing 20 mM lactose; this incubation allowed equilibration of lactose across the lipid bilayer. To establish the background level of nonspecific binding, unloaded samples containing no lactose were employed in some experiments. A 10–20-μl volume of proteoliposomes, containing 0.5 mg of protein/ml, was mixed with 450 μl of phosphate buffer and 0.8 mM of [14C]lactose. A 100-μl sample of this mixture was aliquoted onto a 0.45 μm nitrocellulose filter disc in a vacuum manifold apparatus. The disc was washed several times with phosphate buffer. The amount of [14C]lactose retained on the filter disc was determined by scintillation counting. On co-reconstituted samples, assays were performed either under room lights with additional illumination from a 150-watt tungsten bulb (2000–3000 μE/m2 s) or in the dark (<0.02 μE/m2 s brown-filtered illumination).

**FT-IR Spectroscopy**—Reaction-induced FT-IR spectra on proteoliposomes at pH 7.4 were acquired through the use of a Nicolet 60SXIR spectrometer (18). The mirror velocity was 1.57 cm s \(^{-1}\); the spectral resolution was 8 cm \(^{-1}\), and 360 mirror scans (or 1 min of data collection) were co-added for each single-sided interferogram. A Happ-Genzel apodization function, and one additional level of zero filling were employed. The sample was placed onto a 25-mm CaF\(_2\) window and partially dehydrated under N\(_2\) gas at 4 °C to yield an Amide II absorbance band between 0.25 and 0.60 absorbance units. The relative concentrations of lactose permease and bacteriorhodopsin were measured by monitoring the absorbance at 280 and 565 nm using a Hitachi model U-3000 (Tokyo, Japan) UV-visible spectrophotometer and a clean CaF\(_2\) window as a blank. Following these measurements, the sample was sandwiched with a new and mounted in a Harrick temperature control FT-IR cell. The temperature was maintained at 0 °C. The sample compartment was purged for at least 2 h before data acquisition. Illumination was provided through the use of the 532-nm second harmonic of a pulsed laser (Surelight I, Continuum, Santa Clara, CA), and there was an approximate 0.7-s time delay before the beginning of FT-IR data acquisition. LabView (National Instruments, Austin, TX) software was used to trigger laser pulses and to initiate data collection.

To convert bacteriorhodopsin to the light-adapted form before the beginning of FT-IR data collection, the sample was illuminated with 10 laser pulses at a repetition rate of 1 Hz. After 5 min, data collection began with a set of 5 laser pulses (at 1 Hz), after which five 1-min data sets were collected. This data collection cycle was performed 10–30 times (approximately 1–3 h of data acquisition). Reaction-induced FT-IR spectra were generated by ratioing each 1-min, Fourier processed interferogram to the data obtained 5 min after illumination. In all cases, significant spectral features decayed in less than 5 min after illumination.

Individual difference spectra were averaged and normalized to equal amide II absorbance to correct for alterations in path length and protein concentration. Double difference spectra, reflecting only the contributions of the lactose permease, were generated by subtracting the bacteriorhodopsin difference spectrum. Except where noted, subtraction parameters were determined from the UV-visible spectrum.

In some experiments, 0.5 mM lactose was present in the FT-IR sample. FT-IR samples had the following HEPES concentrations prior to partial dehydration: LP; 10 mM; br; 3, 9, and 19 mM; br + lactose, br + MP, br + LP, br + LP + lactose, 9 mM, br samples, containing 3 mM and 9 mM HEPES, gave reaction-induced spectra that were indistinguishable, given the signal-to-noise.

The FT-IR spectrum of liquid water was acquired through the use of a Nicolet Magna 550 II spectrometer (12) and a Spectra-Tech microcircle cell equipped with a ZnSe crystal. The mirror velocity was 2.5 cm s \(^{-1}\); the spectral resolution was 4 cm \(^{-1}\), and 1-min of data collection was performed at room temperature. A Happ-Genzel apodization function and one additional level of zero filling were employed.

**RESULTS**

Co-reconstitution of bacteriorhodopsin and lactose permease was accomplished with a freeze-thaw method. Functional co-reconstitution was verified using a counterflow assay at pH 6.0 and pH 7.4 (Fig. 1). In this assay, proteoliposomes were pre-loaded with a high concentration of nonlabeled lactose and then were diluted into a solution containing radiolabeled lactose. The data obtained after dilution typically yield a biphasic curve (12). The initial rapid phase of radiolabeled lactose uptake is due to an electroneutral lactose exchange reaction, which is insensitive to changes in the electrochemical gradient (10). A slow phase of H\(^{+}\)/lactose efflux is also seen, and this phase is affected by the H\(^{+}\) electrochemical gradient (11). We have previously shown that proteoliposomes containing the purified permease have low passive permeability to protons and a slow efflux reaction (12).

Co-reconstituted samples containing lactose permease and bacteriorhodopsin exhibited a slow or insignificant rate of efflux when the assay was performed in the dark (Fig. 1, A and B). However, upon illumination of the co-reconstituted sample, the efflux phase of the assay was accelerated (Fig. 1). The stimulation was observed at both pH 6.0 and 7.4 and was similar at the two pH values, given the error bars (Fig. 1, A and B). These data indicate that bacteriorhodopsin pumps H\(^{+}\) into the vesicles and promotes H\(^{+}\)/lactose efflux. This inside-out orientation for bacteriorhodopsin has been observed in other
reconstitution studies (5, 20, 21). Furthermore, because illumination resulted in the efflux of lactose to background levels (Fig. 1, A and B), the results indicate that nearly all permease molecules were reconstituted into vesicles containing bacteriorhodopsin.

The rate of efflux was calculated from the data presented in Fig. 1. The values were in the range from 0.01 to 0.02 lactose (min-permease)$^{-1}$ and are in reasonable agreement with the rate of efflux previously observed at pH 6.0 in the presence of an uncoupler (0.02 lactose (min-permease)$^{-1}$) (12) and observed with a different permease preparation at pH 7.5 (22). These values are much slower than reported rates of proton pumping with a different permease preparation at pH 7.5 (22). These values are much slower than rates previously observed (for example see Ref. 23), suggesting that the permease reactions are rate-limiting.

Fig. 2 presents the results obtained from reaction-induced FT-IR spectroscopy. The light-minus-dark spectra were generated by manipulation of data obtained 1 min following (positive) and 5 min following (negative) laser excitation. As expected, proteoliposomes containing lactose permease alone (LP) gave rise to no defined spectral features (Fig. 2A). Proteoliposomes containing bacteriorhodopsin alone (bR) gave rise to a light-minus-dark difference FT-IR spectrum similar to spectra previously obtained (for example see Refs. 24–26) (Fig. 2B). This spectrum is associated with the isomerization of the retinal chromophore and the deprotonation of bacteriorhodopsin's retinal Schiff base chromophore (reviewed in Ref. 27). A positive band at approximately 1762 cm$^{-1}$ is attributable to the protonation of Asp-85 in the M intermediate, whereas a negative band at approximately 1746 cm$^{-1}$ is attributable to the deprotonation of Asp-96, which occurs in the N intermediate.

![Fig. 2](image-url)
positive bands at 1636 and 1527 cm\(^{-1}\) were still observed (Fig. 3B). Overall, the addition of lactose had a dramatic effect on the spectrum (compare Fig. 3, A and B). As a control, a double difference spectrum (Fig. 3C) was constructed from data obtained from bacteriorhodopsin-containing proteoliposomes in the absence (Fig. 2B) and the presence (Fig. 2C) of 0.5 mM lactose. No defined spectral features were observed (Fig. 3C), supporting the conclusion that lactose has no direct effects on the bacteriorhodopsin photocycle.

Taken together, these experiments suggest that spectral features in Fig. 3A arise from the permease and do not arise from indirect effects of co-reconstitution on bacteriorhodopsin. As an additional control, a double difference spectrum (Fig. 3D) was constructed from data obtained on proteoliposomes in which bacteriorhodopsin was present alone (Fig. 2B) or reconstituted with non-permease containing MP fractions (Fig. 2D). No defined spectral features were observed (Fig. 3D), supporting the conclusion that the subtraction and normalization procedures are effective and that the permease alone contributes to Fig. 3A.

The subtraction method employed to generate Fig. 3 has the advantage that no assumptions are made concerning potential permease contributions to the spectrum. However, more subjective methods can also be applied. In Fig. 4B, the double difference spectrum obtained by a one-to-one subtraction of UV-visible corrected data is repeated from Fig. 3A. In Fig. 4A, bacteriorhodopsin contributions have been over-subtracted in order to minimize spectral contributions at 1527 cm\(^{-1}\). In Fig. 4C, bacteriorhodopsin contributions have been under-subtracted by the same factor. Comparison of Fig. 4, A–C, shows that the spectral region between 1760 and 1670 cm\(^{-1}\) is relatively insensitive to differences in the bacteriorhodopsin subtraction parameter. The positive band at 1734 cm\(^{-1}\) was observed to be nearly invariant in amplitude and frequency (Fig. 4, A–C). The band at 1761 cm\(^{-1}\) was observed at each value of the subtraction parameter but exhibited amplitude variation (Fig. 4, A–C). However, observed spectral differences are on the same order of magnitude as the spectral variation observed over different days of data acquisition and with different co-reconstituted samples (Fig. 4, insets I and II). Therefore, we attribute a positive spectral feature at 1734 cm\(^{-1}\), and possibly 1761 cm\(^{-1}\), to the permease. These conclusions will be tested with isotopic labeling.

Note that although the spectral regions between 2000 and 1660 cm\(^{-1}\) (Fig. 4, inset I) and between 1640 and 1250 cm\(^{-1}\) (Fig. 4, inset II) were similar on different days of data acquisition and with different co-reconstituted samples, the amide I region (1650 cm\(^{-1}\)) did not give sufficiently reproducible results to enable an interpretation of this region of the spectrum. The origin of this spectral variation in the amide I region is under investigation but may be due to variation in protein content and interaction in individual proteoliposomes.

The ratio of amplitudes at 3290 and 1545 cm\(^{-1}\) (Fig. 5, dotted lines) can give a precise estimate of changing water content in these samples, because protein is expected to contribute at both frequencies (31, 32), whereas water contributes significantly only at 3290 cm\(^{-1}\) (Fig. 5A). The average ratio obtained (3290/1545 cm\(^{-1}\)) was 1.3 \(\pm\) 0.5 for the bR samples (Fig. 5B), 1.1 \(\pm\) 0.2 for the bR + LP samples (Fig. 5C), and 1.1 \(\pm\) 0.1 for the bR + MP samples (data not shown). This analysis was consistent with no significant variation in water content when bR, bR + LP, and bR + MP samples were compared. In particular, there was no observed correlation between the 3290/1545 cm\(^{-1}\) ratio and the appearance of spectral features in the double difference spectrum (Fig. 3).

**DISCUSSION**

In order to obtain novel molecular information about transport mechanism, we have employed a new spectroscopic method, reaction-induced FT-IR spectroscopy, to study the lactose permease. Vibrational spectroscopy can yield dynamic information about structural changes occurring in membrane proteins. In our method, lactose permease and bacteriorhodopsin are co-reconstituted, and bacteriorhodopsin is then used as a light-activatable source of a proton electrochemical gradient. Subtraction of bacteriorhodopsin contributions gives a spectrum corresponding to the permease alone. In the resulting
double difference spectrum, the region from 1760 to 1660 cm$^{-1}$ was found to be relatively insensitive to the bacteriorhodopsin subtraction parameter employed. Accordingly, in this report, we focus on a preliminary interpretation of this spectral region.

Positive vibrational lines in Fig. 3A with frequencies between 1765 and 1720 cm$^{-1}$ are assigned to carbonyl stretching bands of glutamic and aspartic acid residues. Aspartic and glutamic acid are the only functional groups in proteins expected to have absorption in this region of the spectrum (35, 34). Because two spectral frequencies may be observed (1761 and 1734 cm$^{-1}$), it is likely that more than one glutamic/aspartic acid residues contributes to the spectrum. An inverse linear correlation of $pK_a$ values and frequency has been observed for certain classes of carboxylic acids (32). Changes in dielectric constant and hydrogen bonding also influence frequency (32, 35).

There are two mechanisms by which carboxylic acids can contribute positive lines to Fig. 3A. The first is that the imposition of the electrochemical gradient causes protonation of one or more carboxylates, and the second is that the electrochemical gradient causes a change in $pK_a$, hydrogen bonding, and/or dielectric constant (32). These two processes have different vibrational signatures. For example, upon protonation of aspartate in vitro, the 1721 cm$^{-1}$ C–O and 1211 cm$^{-1}$ C–O vibrations of the acid replace the 1574 cm$^{-1}$ asymmetric and 1391 cm$^{-1}$ symmetric CO stretching vibrations of the aspartate anion (18). For environmental perturbations or $pK_a$ shifts, a derivative-shaped spectral feature is expected, corresponding to a smaller frequency shift caused by a change in the basicity or double bond character of the C=O bond (32, 35, 36).

One interpretation of Fig. 3A is that negative spectral features at 1692 and, possibly, 1668 cm$^{-1}$ are associated with the positive 1734 cm$^{-1}$ line. This interpretation is consistent with a dramatic change in $pK_a$, or environment, when the electrochemical gradient is applied. Alternatively, the 1668 cm$^{-1}$ and, possibly, the 1692 cm$^{-1}$ line could arise from amide I bands (31). Another possible interpretation of the spectrum is a protonation reaction. Although this interpretation cannot be completely excluded at this time, we do not favor it because the spectrum lacks defined features in the 1400 cm$^{-1}$ region. A definitive assignment of the 1761, 1734, 1692, and 1668 cm$^{-1}$ lines will be performed through the use of isotopic labeling.

In the presence of lactose, the spectrum associated with the imposition of the electrochemical gradient is altered dramatically (Fig. 3B). Given our current signal-to-noise, only two significant spectral features are observed with frequencies at 1636 and 1527 cm$^{-1}$. In the presence of lactose, the overall observation is a decrease in amplitude throughout most of the 2000–1250 cm$^{-1}$ region. This generalized decrease in intensity may be due to the time scale of our measurements. In the absence of lactose, the permease cannot convert between inwardly accessible and outwardly accessible conformations (10). However, when both substrates are available, the permease can cycle through the complete transport process. The turnover number for uphill lactose symport has been reported to be 16–18 s$^{-1}$ in proteoliposomes (37). This turnover rate may preclude the accumulation of reaction intermediates on the time scale employed here. This possibility will be explored in the future through the use of step-scan and rapid-scan FT-IR techniques. A detailed interpretation of Fig. 3B awaits these future studies.

The results of our spectroscopic study indicate that the electrochemical gradient perturbs aspartic and glutamic acid residues in the permease. This perturbation may correspond to an alteration in hydrogen bonding, dielectric constant, $pK_a$, or protonation state. The observation that the electrochemical gradient perturbs carboxylic acid residues is consistent with previous work showing that mutations of carboxylates at positions 126, 240, 269, and 325 block the active accumulation of lactose (see Refs. 3, 4 and references therein). A change in $pK_a$ or net protonation reaction could be involved in the storage of free energy derived from the electrochemical gradient. This change in $pK_a$ and/or protonation could be a direct result of the proton gradient or could be an indirect effect caused by the generation of positive charge or by helix dipole rearrangements.

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