**Lifetime and Rotational Relaxation Time of Dansylgalactoside Bound to the Lac Carrier Protein**

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The results presented in this paper demonstrate that the excited state lifetime, anisotropy, and rotational relaxation time of 2'-(N-dansyl)aminoethyl 1-thio-\(\beta\)-D-galactopyranoside (DG\(_2\)) increase when the probe is bound specifically to the lac carrier protein in "energized" Escherichia coli membrane vesicles. Although the probe also binds nonspecifically to the vesicle membrane, such binding is independent of the lac carrier protein and is unaffected by "energization" of the vesicles. The experiments provide further evidence that the dansylgalactosides are useful probes for the \(\beta\)-galactoside transport system and support the hypothesis that the changes in dansylgalactoside fluorescence observed on "energization" of membrane vesicles reflect changes in the binding of the probe.

Fluorescence studies with (N-dansyl)aminoalkyl 1-thio-\(\beta\)-D-galactopyranosides (1-3) and photoinactivation studies with (2-nitro-4-azido)-\(\beta\)-D-galactopyranosides (4, 5) indicate that the lac carrier protein in membrane vesicles isolated from Escherichia coli does not bind ligand in the absence of a membrane potential (interior negative). As a result of D-lactate or reduced phenazine methosulfate oxidation, or artificially induced ion gradients, an electrical potential is generated across the vesicle membrane (6-9), and changes in dansylgalactoside fluorescence and azidophenylgalactoside-dependent photoinactivation are observed. Alternatively, dilution-induced, carrier-mediated lactose efflux also causes changes in dansylgalactoside fluorescence by a mechanism which apparently functions independently of the membrane potential (2). Based on these studies, it has been postulated (2) that the membrane potential causes the lac carrier protein to become accessible to the external medium, to increase its affinity for ligand, or both; and it has been suggested that the lac carrier protein or a part of it may be negatively charged. The veracity of these conclusions depends on the contention that the fluorescent changes observed with the dansylgalactosides are due specifically to binding, and this aspect of the problem has been approached in several ways. It has been demonstrated that there is a marked increase in polarization of dansylgalactoside fluorescence on energization of ML 308-225 membrane vesicles (2), that the dansylgalactosides are not transported to any demonstrable extent (1-3), and that the changes in dansylgalactoside fluorescence observed on energization cannot be attributed to binding followed by translocation into the hydrophobic milieu of the membrane (1, 3).

The data presented in this paper provide further support for these conclusions by means of lifetime and anisotropy studies. The experiments demonstrate that the lifetime of the excited state of 2'-(N-dansyl)aminoethyl 1-thio-\(\beta\)-D-galactopyranoside (DG\(_{1}\)) is increased when the molecule is bound to the lac carrier protein and concurrently the rotational diffusion of DG\(_{1}\) is dramatically decreased. Direct binding measurements with 6'-(N-[\(\beta\)]dansyl)aminohexyl 1-thio-\(\beta\)-D-galactopyranoside (DG\(_{4}\)) will be presented in another publication.

**EXPERIMENTAL PROCEDURE**

**Growth of Cells and Preparation of Membrane Vesicles**

*Escherichia coli* ML 3 (\(i^{+}z^{+}y^{-}a^{+}\)) and ML 308-225 (\(i^{+}z^{-}y^{+}a^{-}\)) were grown on minimal medium A with 1.0% sodium succinate (hexahydrate) and membrane vesicles were prepared as described elsewhere (10).

**Fluorescence Measurements**

Polarization of Fluorescence—Polarization measurements were made as described elsewhere (2) in mechanically stirred thermostated cuvettes (1 x 1 cm). The excitation light for the polarization experiments was defined at 340 nm by the monochromator, and a Glan-Taylor prism polarizer (Karl Lambrecht Corp., CTYA 12). The analyzer was set to transmit vertically polarized light. The right angle emission was defined by a blocking filter combination of a Corning 3-75 filter, a sodium nitrite filter, and an analyzer made of a glass-enclosed polaroid sheet. The analyzer filter was adjusted to measure light polarized horizontally (i.e. perpendicular (\(\perp\)) to the vector of the incident beam) or vertically (i.e. parallel (\(\parallel\)) to the vector of the incident beam). A is the anisotropy of the system and is defined as:

\[ A = \frac{I_{\perp}}{I_{\parallel} + 2I_{\perp}} \]

where \(I_{\perp}\) and \(I_{\parallel}\) are fluorescence intensities observed through a

\[ \perp \text{ and } \parallel \text{ are perpendicular and parallel to the vector of the incident beam.} \]

\[ 1 \text{ The abbreviations used are: DG}_{1}, 2-(N-dansyl)aminoethyl 1-thio-\(\beta\)-D-galactopyranoside; DG}_{4}, 6'-(-N-[\(\beta\)]dansyl)aminohexyl 1-thio-\(\beta\)-D-galactopyranoside; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.} \]


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polarizer oriented parallel and perpendicular, respectively, to the plane of polarization of the excitation beam.

In order to calculate the individual contributions of specific polarized species in solution, the law of addition of anisotropies was applied (11–13):

\[ A = \sum_i A_i f_i \]

where \( f_i \) is the fraction of the total intensity emitted by the \( i \)th component. The \( f_i \) values were obtained from the lifetime measurements, and they were similar to those obtained from the increase in fluorescence intensity under the conditions used.

**Lifetime Measurements**—The fluorescence lifetimes (\( \tau \)) were determined from both phase and modulation measurements on the sub-nanosecond cross-correlation spectrofluorometer of Spencer & Weber (14, 15) using exciting light modulated at 14.2 MHz and 28.4 MHz. With this technique, the independent measurements of phase (\( \Phi \)) and modulation (\( M \)) reflect the fluorescence lifetime by the laws:

\[ \tau = \frac{\arctan \Phi}{2\pi f} \]  

\[ \tau = \sqrt{1/M^2 - 1/2\pi f^2} \]  

If the emission is spectrally homogeneous (one exponential decay), then Equations 1 and 2 give identical lifetime calculations within the standard deviation of the measurements of phase and modulation. The demonstrated standard deviation for the range of 0 to 20 ns on this instrument is \( \pm 0.05 \) ns.

If Equations 1 and 2 do not yield the same lifetime from the phase and modulation measurements, then two or more components may be present and the following equations apply:

\[ \tan \Phi = -\frac{(\Sigma f_i \sin \Phi_i \cos \Phi_i)/\Sigma (f_i \cos^2 \Phi_i)}{\Sigma (f_i \cos^2 \Phi_i)} \]  

\[ \mathcal{M}^2 = \frac{\Sigma (f_i \sin \Phi_i \cos \Phi_i)^2 + \Sigma (f_i \sin \Phi_i \cos \Phi_i)^2}{\Sigma (f_i \sin \Phi_i \cos \Phi_i)^2} \]  

where \( \Phi \) and \( \mathcal{M} \) are the experimental phase and modulation values, and \( \Phi_i \) and \( f_i \) are the phase shift and fraction of the \( i \)th component. These equations were used in an iterative program on a Compucorp 322 programmable calculator to match a two component decay system to the experimental phase and modulation measurements and to verify the lack of a significant third component (16).

The instrument can make measurements rapidly enough to follow the kinetics of interactions at 10 sec intervals.

**Calculation of Rotational Relaxation Time**—The rotational relaxation time of a molecule, \( \rho \), is defined as the average time required for the molecule to be displaced from its original orientation by a mean angle of arc \( \cos^{-1} \). The anisotropy of a molecule relates to its rotational relaxation time.

\[ A_0/A = 1 + 3\rho/\rho \]

where \( A_0 \) is the limit fluorescence anisotropy, \( A \) is the measured fluorescence anisotropy, \( \tau \) is the lifetime of the excited state, and \( \rho \) is the rotational relaxation time.

**Materials**—2′-(N-dansyl)aminoethyl 1-thio-\( \beta \)-d-galactopyranoside (DG\(_1\)) and 6′-(N-dansyl)aminoethyl 1-thio-\( \beta \)-d-galactopyranoside (DG\(_2\)) were synthesized as described previously (2). All other materials were of reagent grade obtained from commercial sources.

**RESULTS**

Lifetime Measurements—As shown in Table I, the fluorescence lifetime of 2′-(N-dansyl)aminoethyl 1-thio-\( \beta \)-d-galactopyranoside (DG\(_1\)) in potassium phosphate buffer at pH 6.6 is 3.0 ns when measured by either phase or modulation as described under “Experimental Procedure.” The identity of the excited state lifetime of DG\(_1\), as determined by these two measurements provides strong evidence for spectroscopic homogeneity (14–16). In the presence of *Escherichia coli* ML 308-225 membrane vesicles, the lifetime of DG\(_1\), increases to 3.94 ns as determined by phase measurements and 6.65 ns by modulation measurements. As described under “Experimental Procedure,” the discrepancy in the lifetime as determined by these two methods can be shown by computer analysis to be due to the presence of two species in the reaction mixture each of which has a different excited state lifetime: (a) a new species of DG\(_2\) which has a lifetime of 18 ns and accounts for 18% of the total signal (Table I); and (b) the remainder of the signal (i.e. 82%), accounted for by the free DG\(_2\) which continues to exhibit a lifetime of 3.0 ns. This increase in lifetime observed in the presence of ML 308-225 vesicles is apparently nonspecific since an almost identical increase in lifetime is observed in the presence of ML 3 lac carrier vesicles which are devoid of lac carrier protein. As shown, on addition of ML 3 vesicles, virtually the same increase in lifetime is observed as in the case for ML 308-225 vesicles, and 17% of the signal can be accounted for by a species with a lifetime of 18 ns. Moreover, addition of lactose, the physiologic substrate of the lac carrier protein, at a concentration 40 times higher than the \( K_m \) of the transport system (17), does not alter the lifetime of DG\(_2\) with either of the vesicle preparations.

When D-lactate is added to the cuvette containing ML 308-225 vesicles, the lifetime of DG\(_2\) as determined by phase measurements increases to 4.5 ns (Table I). Modulation measurements carried out on the same sample yield a value of 7.8 ns. The results are compatible with a two-component system in which the proportion of the DG\(_2\) species exhibiting a lifetime of 18 ns increases from 18% to 27% of the total signal intensity. Moreover, the increase observed on addition of D-lactate is due to specific binding to the lac carrier protein since: (a) no increase in the phase and modulation lifetimes of

**TABLE I**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Consecutive addition</th>
<th>(\Phi)</th>
<th>(\tau_p)</th>
<th>(f_1)</th>
<th>(t_1)</th>
<th>(f_2)</th>
<th>(t_2)</th>
<th>(f_3)</th>
<th>(t_3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ML 308-225</td>
<td>DG(_2), 50 (\mu)M + membrane vesicles, 0.4 mg of protein/ml + Li-D-lactate, 20 (\mu)M + Lactose, 10 (\mu)M</td>
<td>3.86</td>
<td>6.45</td>
<td>0.825</td>
<td>3.0</td>
<td>0.18</td>
<td>17.2</td>
<td>0.19</td>
<td>17.5</td>
</tr>
<tr>
<td>ML 3</td>
<td>DG(_2) + membrane vesicles, 0.4 mg of protein/ml + D-lactate</td>
<td>3.88</td>
<td>6.5</td>
<td>0.82</td>
<td>3.0</td>
<td>0.18</td>
<td>18</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
of vesicles, it can be calculated that the anisotropy of the nonspecifically bound DG₄ is 0.113 (cf. "Experimental Procedure"). When D-lactate is added to the cuvette containing ML 308-225 vesicles, the measured anisotropy increases to 0.0696. When D-lactate is added to ML 3 vesicles in the presence of DG₄, no increase in anisotropy is observed (not shown). Thus, the increase from 0.0426 to 0.0696 resulting from the addition of D-lactate to ML 308-225 vesicles is due to specific binding of DG₄ to the lac carrier protein. Using the law of addition of anisotropies (cf. "Experimental Procedure") and \( f_i \) values determined from lifetime measurements and assuming that the nonspecifically bound fraction remains constant, it can be calculated that the specifically bound DG₄ exhibits an anisotropy value of 0.291. The limiting anisotropy of DG₄ measured in 100% glycerol at -20°C is 0.396. It is also noteworthy that qualitatively similar results were obtained when these studies were carried out with 6'-(N-dansyl)aminohexyl 1-thio-β-D-galactopyranoside (DG₄) (data not shown).

### Rotational Relaxation Time

It follows directly from the results discussed above that the rotational rate of the nonspecifically bound DG₄ is much greater than that of DG₄ bound to ML 3 vesicles, therefore the rotational rate of DG₄ in the presence of ML 3 vesicles is due to specific binding of DG₄ to the lac carrier protein. Using the law of addition of anisotropies (cf. "Experimental Procedure") and \( f_i \) values determined from lifetime measurements and assuming that the nonspecifically bound fraction remains constant, it can be calculated that the specifically bound DG₄ exhibits an anisotropy value of 0.291. The limiting anisotropy of DG₄ measured in 100% glycerol at -20°C is 0.396. It is also noteworthy that qualitatively similar results were obtained when these studies were carried out with 6'-(N-dansyl)aminohexyl 1-thio-β-D-galactopyranoside (DG₄) (data not shown).

### Calculation of Fluorescence Anisotropy of D-galactosides Bound to lac Carrier Protein

**Table II**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Additions</th>
<th>( A_{\text{exp.}} )</th>
<th>( f_i )</th>
<th>( A_i )</th>
<th>( f_i )</th>
<th>( A_i )</th>
<th>( f_i )</th>
<th>( A_i )</th>
</tr>
</thead>
<tbody>
<tr>
<td>ML 308-225</td>
<td>DG₄, 90 μM</td>
<td>0.0272</td>
<td>1.0</td>
<td>0.0272</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ Membrane vesicles, 0.4 mg/ml</td>
<td>0.0426</td>
<td>0.82</td>
<td>0.0272</td>
<td>0.18</td>
<td>0.113</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ D-lactate, 20 mM</td>
<td>0.0696</td>
<td>0.73</td>
<td>0.0272</td>
<td>0.16</td>
<td>0.113</td>
<td>0.11</td>
<td>0.291</td>
</tr>
</tbody>
</table>
the lac carrier protein. Thus, the relationship between the fluorescence anisotropy of the system and the rotational diffusion of the probe can be used to further characterize the different species of DG, present under the various conditions studied. The rotational relaxation times shown in Table III were calculated using the Perrin equation (cf., "Experimental Procedure") and the lifetime and anisotropy values obtained from Tables I and II, respectively. The rotational relaxation time, \( \rho \), of DG, in potassium phosphate buffer at pH 6.6 is approximately 0.66 ns. This value increases approximately 30-fold to 22 ns when DG, is nonspecifically adsorbed to the vesicle membrane (i.e., ML 308-225 vesicles in the absence of \( \beta \)-lactate or ML 3 vesicles in the presence or absence of \( \beta \)-lactate) and almost 230-fold when DG, is bound to the lac carrier protein (i.e., when \( \beta \)-lactate is added to ML 308-225 vesicles).

**DISCUSSION**

The data presented in this paper provide strong support for previous observations (1–3) related to the use of dansylgalactosides as specific probes for the \( \beta \)-galactoside transport system in *E. coli* membrane vesicles. As shown previously, no changes in the fluorescence of various dansylgalactosides are observed unless vesicles containing the lac carrier protein are "energized" by means of a membrane potential (interior negative) generated by either \( \beta \)-lactate oxidation or artificially imposed ion gradients. Similarly, in these studies, no changes in the excited state lifetime, anisotropy, or rotational relaxation time of DG, which can be attributed to specific binding to the lac carrier protein are observed unless the appropriate vesicles are "energized" by means of \( \beta \)-lactate oxidation. Moreover, when \( \beta \)-lactate is added to vesicles containing the lac carrier protein, increases in all three parameters are observed. In addition to providing corroboration for the previous findings, the results are also consistent with the conclusion (1–3) that the fluorescence changes observed on "energization" of the vesicles reflect changes in binding *per se* rather than binding followed by translocation into the hydrophobic interior of the membrane.

The use of more sophisticated fluorescence techniques has also allowed the detection of nonspecific binding of dansylgalactoside to the vesicle membrane. This nonspecific component which gives rise to the heterogeneity in the lifetimes and is also evident in the anisotropy measurements cannot be demonstrated by spectral differences, which would be small and distorted by the presence of the vesicles.

It is noteworthy that nonspecifically bound DG, as well as DG, bound specifically to the lac carrier protein exhibits the same excited state lifetime of 18 ns. This value is quite high and close to the maximal lifetime reported for the dansyl fluorophore which is approximately 20 ns (20). The finding indicates that although the environment of the probe may differ when it is bound specifically versus nonspecifically, such differences would not be detected because both species exhibit almost maximal lifetimes. In marked contrast to the similarity of the lifetimes of specifically and nonspecifically bound DG, the rotational rate of DG, bound specifically to the lac carrier protein is much less than the rotational rate of the nonspecifically bound species. The 150-ns rotational relaxation time of the specifically bound probe is, in fact, comparable to analogous values for fluorophores covalently bound to a globular protein of 100 to 150 kilodaltons.

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**REFERENCES**

Lifetime and rotational relaxation time of dansylgalactoside bound to the lac carrier protein.
S Schuldiner, R D Spencer, G Weber, R Weil and H R Kaback


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