The Mechanism of Ligand Binding to the Periplasmic C4-dicarboxylate Binding Protein (DctP) from *Rhodobacter capsulatus*  

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The kinetics of ligand binding to the periplasmic C4-dicarboxylate binding protein (DctP) from *Rhodobacter capsulatus* were investigated by exploiting the changes in the intrinsic fluorescence of the protein upon binding ligands. Steady state measurements have shown that ligands such as malate, succinate, and fumarate are all bound with sub-micromolar *K*ₐ values, whereas D-malate is bound 2 orders of magnitude more weakly. Stopped-flow studies revealed that the binding process involves at least three steps. In the absence of ligand, the protein is in equilibrium between an essentially nonbinding form, BP₁, and the binding form, BP₂. Ligands bind to the BP₂ form, shifting the equilibrium toward the BP₂-¹ form, and inducing a further isomerization of the protein, to the BP₂-¹ form. The kinetic properties of the four different conformational states of the DctP protein identified in this study would be consistent with their identification as the closed-conformation, the open-conformation, an open-liganded conformation, and a closed-liganded conformation. The latter three states have been identified by x-ray crystallographic studies of binding proteins, but no kinetic or structural data have been presented previously to support the possibility of a closed but unliganded conformation.

Periplasmic permeases form an important class of bacterial solute transporters (Ames, 1986, 1988). An essential component of this type of transport system is the substrate binding protein, which is released into the periplasmic space of Gram-negative bacteria in order to sequester the ligand to be transported (Willis et al., 1974; Quiocio, 1990). Subsequently, this protein interacts with a complex of three or four membrane-associated proteins, which elicit the translocation of the ligand across the membrane. This complex generally consists of two hydrophobic integral membrane proteins and one or two hydrophilic membrane-associated proteins (Higgins et al., 1990). The latter proteins are involved in binding ATP, the hydrolysis of which is believed to drive ligand translocation (Minnick et al., 1989; Ames et al., 1989; Prossnitz et al., 1989).

Of the various protein components involved in this type of transport system, the periplasmic binding protein has been characterized in the most detail. Several of these have been purified to homogeneity and their structures determined by x-ray crystallography, with and without bound ligand (Quiocio et al., 1977; Sack et al., 1989a, 1989b; Quiocio, 1990). These studies have revealed a common structural unit within this group of proteins, although there is little primary sequence homology between them. Basically, the structure consists of two globular domains connected by a hinge region. Ligand binding occurs between the two domains, displacing bound water molecules from both the ligand and the protein. These crystallographic studies have indicated that the protein can adopt at least three different conformational states; an open form, in which the two globular domains are apart, an open-liganded form, and a closed-liganded form, in which the two globular domains have encompassed the ligand. The demonstration of the existence of these three conformational states has been interpreted in terms of a model for ligand binding (Mao et al., 1982). This model proposes that the binding protein exists solely in the open conformation and that ligand binding triggers closure of the two globular protein domains around the ligand; the so-called "Venus flytrap" model. To some extent, this model has been supported by kinetic studies on amino acid, sugar, and inorganic ion binding proteins, which have provided evidence for a single-step equilibrium binding process (Miller et al., 1980, 1983). The fact that the apparent association rate constants for these proteins are 1 to 2 orders of magnitude slower than predicted for a diffusion controlled process has been interpreted as due to a conformational change in the protein following the initial encounter of the ligand with the protein (Miller et al., 1983).

In contrast to the well characterized periplasmic permeases for amino acids, sugars, and inorganic ions, relatively few studies have been made of the corresponding transport systems for organic acids. A binding protein-dependent tricarboxylate transport system has been characterized in *Salmonella typhimurium* (Widenhorn et al., 1988) and the binding protein itself (TctC) has been crystallized (Sweet et al., 1984). Binding protein-dependent C4-dicarboxylate transport has been studied in *Escherichia coli* (Lo and Sanwal, 1975) and in the purple photosynthetic bacterium *Rhodobacter capsulatus* (Hamblin et al., 1990; Shaw and Kelly, 1991). In the latter case, the binding protein has been purified and the corresponding gene (dctP) cloned and sequenced (Shaw et al., 1991). The mature form of DctP is a 307-residue protein with a molecular weight of 33,567, which binds malate, succinate, and fumarate as physiological substrates. In this paper, we have used fluorescence spectroscopy to study the ligand-binding properties of DctP. Using a stopped-
flow technique to time-resolve the binding process, we have obtained evidence for a three step binding mechanism, involving a slow isomerization of the protein between non-binding and binding forms, followed by a rapid binding phase, and a further isomerization. Such a mechanism has not previously been reported for a periplasmic binding protein.

RESULTS

Fluorescence Properties of the Binding Protein—The protein fluorescence was characterized by excitation and emission peaks centered at 280 and 319 nm, respectively (Fig. 1). The excitation peak at 280 nm probably reflects the high ratio of tyrosine to tryptophans within the protein. Sequence analysis of the dctP gene (Shaw et al., 1991) has revealed that there are 9 tyrosine and 3 tryptophan residues. The emission spectra would suggest that the tryptophans contributing to the protein fluorescence are located largely within a hydrophobic environment. Indeed, increasing the excitation wavelength to 297 nm, to excite the tryptophans selectively, caused a shift in the emission maxima to around 327 nm, still indicative of a hydrophobic environment. Consistent with this interpretation we found that the protein fluorescence was only quenched by about 24% with 125 mM potassium iodide, a hydrophilic collisional quencher. Interestingly, however, in the presence of KI, the emission peak was red shifted to around 326 nm. This is unexpected since KI should preferentially quench those residues which lie within a hydrophilic environment, thus blue-shifting the spectrum. One possible explanation is that the KI is also quenching those tyrosine residues that lie in hydrophilic environments. A Stern-Volmer plot of the KI titration data for the binding protein was downwardly curved (data not shown), indicating that there is a heterogeneous population of tryptophan residues, differing in their ability to be quenched. Accordingly, these data were analyzed in terms of the modified Stern-Volmer equation, which allows for accessible and inaccessible tryptophan residues (Lehrer and Leavis, 1978),

\[ F_0/(F_0 - F) = 1/K_f K + 1/F_0 \]

where \( F_0 \) is the fluorescence in the absence of quencher, \( F \) the fluorescence after the addition of quencher to a concentration \( [Q] \), \( F_0 \) is the effective maximal fractional of the fluorescence accessible to the quencher at infinite quencher concentration, and \( K \) is the Stern-Volmer quenching constant. A plot of \( F_0/(F_0 - F) \) versus \( 1/[Q] \) was linear with a y intercept of \( 1/F_0 \) and slope of \( 1/K_f K \), giving values for \( F_0 = 0.37 \) and \( K = 64.8 \text{ M}^{-1} \) (Fig. 2). The modified Stern-Volmer plot thus indicated that 37% of the tryptophans are quencher accessible in DctP.

Ligand binding was also investigated by fluorescence spectroscopy (Fig. 1). Fumarate was found to have the largest effect upon the proteins fluorescence, with 100 \( \mu \)M fumarate quenching the fluorescence of 4 \( \mu \)M protein by 17%, without shifting the emission maximum. In contrast, succinate induced about a 4.5% fluorescence enhancement, whereas L- and D-malate induced about a 2% enhancement, again without affecting the emission maxima (Fig. 1). Pyruvate, which does not inhibit \( ^{14}\text{C} \)-L-malate binding (Shaw et al., 1991), had no effect on the fluorescence emission spectrum. The protein was titrated with KI in the presence of saturating concentrations of fumarate and L-malate in order to ascertain whether these ligands could be shown to be supporting different conformational states. In the presence of fumarate \( f_3 = 0.31 \) and
Ligand Binding to the DetP Protein of Rb. capsulatus

Fig. 2. Modified Stern-Volmer plots of $F_0/(F_0 - F)$ versus $1/[K_I]$ for C4-dicarboxylate binding protein (4 µM) in the absence of ligand (○—○) or 100 µM fumarate (△—△). $F_i$ is the fluorescence of the protein in the absence of $K_I$ and $F$ the fluorescence after addition of $K_I$ to the indicated concentration. In the presence of ligand, $F_0$ was taken as the fluorescence after addition of the ligand.

$K = 65.5$ M$^{-1}$, whereas for L-malate $F_i = 0.42$ and $K = 66.9$ M$^{-1}$ (Fig. 2). The moderate difference in the $K_I$ quenching parameters for these two ligands would suggest that they are supporting different conformational states.

The ligand binding stoichiometry of the protein was determined by titrating the protein with fumarate. The titration data are shown in Fig. 3. The shape of this plot is highly indicative of tight binding. Under such conditions the data will provide an accurate measure of the stoichiometry for ligand binding. Extrapolation of the limiting asymptotes of the data indicated a stoichiometry of 1 mol of fumarate binding per mol of protein. An estimate of the $K_f$ was obtained from a fit of the data in Fig. 3 to an equation describing the titration curve for a second order binding process,

$$-F/F_0 = \frac{(\Delta F/(F_0))(K_f + [BP] + [L])}{K_f + [BP] + [L]}$$

$$- \frac{\sqrt{[K_f + [BP] + [L]^2 - 4[BP][L]]}}{2}$$

where $BP$ and $L$ represent the binding protein and ligand concentrations, $F_0$ and $\Delta F$ are the initial and total fluorescence changes, and $K_f$ the dissociation constant. Such an analysis yielded a lower limit for the fumarate dissociation constant of 0.255 µM. The data would not be expected to yield an accurate measure of the dissociation constant due to the tight binding. Indeed, under such circumstances the apparent dissociation constant will depend upon the protein concentration and will tend to decrease with increasing protein concentration. However, reducing the protein concentration used for the titration from 4 to 0.5 µM did not appreciably change the dissociation constant obtained, indicating that it must lie between 0.25 and 0.5 µM. A further reduction in the protein concentration was not possible due to the loss in signal amplitude.

The ligand dissociation constants for the binding of L- and D-malate and succinate directly because of the small fluorescence change associated with the binding of these ligands. Instead, the protein was saturated with fumarate (4 µM protein mixed with 5 µM fumarate) and then the protein was titrated with each ligand, observing the increase in protein fluorescence as the fumarate was displaced from the protein. This procedure allowed the signal change associated with ligand binding to be maximized sufficiently to allow accurate titration of the apparent $K_f$. Assuming that there is simple competition between the fumarate and the ligand for the binding site of the protein, the dissociation constant for each ligand can be calculated from the following relationship,

$$K_f = \frac{K_{app}}{(1 + [fumarate]/K_d)}$$

where $K_{app}$ and $K_f$ are the respective dissociation constants for the ligand and fumarate, and $K_{app}$ is the apparent dissociation constant. In each case the $K_{app}$ could be accurately obtained from a hyperbolic fit of the titration data. This was possible because using 5 µM fumarate gives an apparent $K_{app}$ some 20-fold greater than the true $K_f$ for the ligand under investigation. The calculated values for each ligand dissociation constant are tabulated in Table I. Although these values may not be exact, since they are dependent upon the value attributed to the fumarate dissociation constant, they usefully reveal the order of ligand binding affinity for the protein and establish their relative magnitudes. L-malate was found to have the highest affinity, being more than 2 orders of magnitude greater than D-malate, which had the lowest affinity. Fumarate and succinate had similar affinities to one another, which were less than 1 order of magnitude lower than L-malate.

Ligand Binding Kinetics—The kinetics of ligand binding to the periplasmic binding protein were investigated by stopped-flow fluorescence spectroscopy. We found that the changes in the intrinsic fluorescence of the protein could be usefully exploited in determining the kinetics of ligand binding for a

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K_d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fumarate</td>
<td>0.255</td>
</tr>
<tr>
<td>L-Malate</td>
<td>0.05</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.17</td>
</tr>
<tr>
<td>D-Malate</td>
<td>6.3</td>
</tr>
</tbody>
</table>

TABLE I

Ligand dissociation constants

The fumarate $K_a$ was measured directly by titration of the protein fluorescence with the ligand. The $K_a$ was determined by fitting the titration data to a quadratic equation defining a second-order binding process. The $K_d$ values of the other three ligands were determined by titration of the protein fluorescence of the protein-fumarate complex: apparent $K_a$ values were determined by fitting the titration data to a hyperbola and then correcting to the true $K_a$ values using the formula:

$$R = K_{app}/(1 + [fumarate]/K_d),$$

where $R$ is the $K_a$ obtained for fumarate.
series of potential substrates, including fumarate, D- and L-malate, and succinate.

Fumarate Binding—Fumarate binding was characterized by a moderately fast decrease in protein fluorescence, which amounted to a signal change of about 14% with near saturating fumarate (see Fig. 4). The rate of signal change was readily obtained from a single exponential fit of the data. However, toward the end of the traces there was a slight downward drift in fluorescence. To establish whether this slow phase was due to a further conformational change in the protein, two experiments were performed in which the protein fluorescence was monitored over a 500-s period after mixing with 200 μM fumarate in the stopped-flow spectrometer. In one experiment, the arc lamp shutters were left open during the course of the experiment and in the other they were closed after the initial mixing and re-opened again immediately prior to the end of the experiment. The slow drift in the protein fluorescence was only noted for the protein which had been left in the light, establishing that this fluorescence change is due to photobleaching, rather than any slow conformational change. Furthermore, fitting the data to either a double exponential function or a single exponential with a linear component gave only marginally better fits over the investigated [fumarate] range, as assessed by the change in residual variance of the data (Table III). This would be expected for fits which had an increased degree of freedom due to the additional fitting parameters. However, considering that there is a slow drift in the fluorescence of the protein due to photobleaching, the latter function would appear to be the most appropriate for analyzing the data and was used in analyzing subsequent traces.

Comparison of the stopped-flow traces obtained when 4 μM protein was mixed with 200 μM fumarate with those obtained when the protein was mixed with the buffer alone indicated that there was an initial rapid quench in the protein fluorescence (see Fig. 4). This observation is consistent with our finding that the apparent total quench in protein fluorescence (the recorded signal change) in our stopped-flow studies was consistently found to be less than that obtained from steady state fluorescence measurements using the spectrofluorimeter (up to about 1–3% difference in fluorescence). The kinetics of this phase could not be analyzed in detail since it was of very small amplitude and occurred with a rate beyond the time resolution of the stopped-flow spectrometer.

The [fumarate] dependence of the binding phase is illustrated in Fig. 5, in which the rate is shown to decrease hyperbolically. This kinetic behavior would not be consistent with the simple association of ligand and protein, where one would expect to observe an increase in the rate. However, such behavior could be explained in terms of a slow isomerization of the protein, due to ligand binding predominantly to one form of an equilibrium mixture of two pre-existing protein conformations. Assuming ligand binding to be a rapid equilibrium process compared with the rate of conformational change, the rate constant for the apparent binding phase would be a single exponential function, with a rate that decreased with increasing ligand concentration, according to the equation defining Scheme I.

\[
BP_1 \xrightarrow{k_1} BP_2 \xrightarrow{k_2} BP - L
\]

SCHEME I

\[
k_{obs} = k_1 + k_{-1} \frac{[L]}{[L] + [BP_1]}
\]

TABLE II
Comparison of the nonlinear regression fits obtained using different fitting equations applied to a single stopped-flow trace for fumarate binding

<table>
<thead>
<tr>
<th>Fitting equation</th>
<th>Rate</th>
<th>Amplitude</th>
<th>Residual variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single exponential</td>
<td>(k_1 = 6.24 (\pm 0.031))</td>
<td>100</td>
<td>1.16 \times 10^{-4}</td>
</tr>
<tr>
<td>Double exponential</td>
<td>(k_1 = 7.00 (\pm 0.101)), (k_2 = 2.25 (\pm 0.615))</td>
<td>88.5</td>
<td>0.94 \times 10^{-4}</td>
</tr>
<tr>
<td>Single exponential + drift</td>
<td>(k_1 = 6.5 (\pm 0.041)), slope = 0.014 (\pm 1.8 \times 10^{-5})</td>
<td>98.9</td>
<td>0.97 \times 10^{-4}</td>
</tr>
</tbody>
</table>

* The amplitude of the exponential phase was normalized to the total amplitude for a single exponential fit.
The appearance of the DctP-fumarate complex can be interpreted in terms of the rate constant, $k_\text{-}f$, for the dissociation of the complex. We found that the resulting increase in protein concentration of the displacing ligand would decrease with increasing $\text[fumarate]$. Then the apparent binding rate would decrease with increasing concentration of the displacing ligand.

In order to distinguish between these two possible models the fumarate dissociation constant was determined directly in a ligand displacement type experiment. Under the conditions used for these experiments Scheme II would necessarily prevail. The protein (4 $\mu M$) was pre-equilibrated with 10 $\mu M$ fumarate and this was displaced from the protein by mixing with 2 $M$ L-malate (Fig. 6). According to Scheme II, if $k_\text{-}f \ll k_\text{[L-malate]} \gg k_\text{[fumarate]}$, the signal due to the disappearance of the DctP-fumarate complex can be interpreted in terms of the rate constant, $k_\text{-}f$ ($k_\text{diss}$), for the dissociation of the complex. We found that the resulting increase in protein fluorescence occurred with a rate of 11.7 s$^{-1}$. The lower record (B) shows the decrease in protein fluorescence when 4 $\mu M$ DctP was mixed with 200 $\mu M$ fumarate. Fitting the data to a single exponential function indicated a rate of 8.4 s$^{-1}$. The standard errors of the fitted rates are less than 10%.

Indeed, a visual inspection of Fig. 5 indicates that the dissociation rate should be greater than 100 s$^{-1}$. Clearly then, the data are not consistent with such a model. However, this behavior is consistent with a slow rate-limiting isomerization of the protein, as described by Scheme I.

Since our interpretation of the binding data is critically dependent upon whether or not there is any endogeneous ligand, the protein was subjected to reversible denaturation with urea in order to remove any bound ligand. The renatured protein had a spectrum identical to the native protein, suggesting that no ligand remained associated with the protein after purification. However, since saturating L-malate only appears to produce about a 2% enhancement in fluorescence, any L-malate remaining bound to the protein following the urea treatment might go unnoticed. To assess this possibility the protein was equilibrated with 100 $\mu M$ fumarate and then treated with urea. We found that following the urea treatment, the 17% quench in protein fluorescence caused by fumarate binding was completely relieved, but could be restored again by a further addition of fumarate. These results would indicate that the urea treatment was capable of removing any endogenously bound ligand by reversibly denaturing the protein.

Fumarate binding to the urea-treated protein was investigated. Titration of the protein fluorescence with fumarate indicated that it bound with a stoichiometry of 1 and with a similar dissociation constant ($K_d = 0.43 \pm 0.165 \mu M$) to that obtained for the native protein ($K_d = 0.26 \pm 0.079 \mu M$). As shown in Fig. 7, the stopped-flow traces for fumarate binding to both the native and urea-treated protein are essentially identical. The concentration dependence for fumarate binding to the urea-treated protein was determined, and both the rate and amplitude were found to have the same dependencies as for the native protein (data not shown). Again, displacement of bound fumarate from the protein by an excess of L-malate then the apparent binding rate would decrease with increasing concentration of the displacing ligand.

![Fig. 5. The fumarate dependence of the apparent binding rate. The curve through the data points represents the best fit of the data according to Equation 4. The quoted concentrations are for those in the mixing chamber of the stopped-flow spectrometer.](image)

![Fig. 6. Comparison of the stopped-flow records showing fumarate binding to DctP and L-malate displacement of fumarate from DctP. The upper record (A) shows the increase in protein fluorescence when 4 $\mu M$ DctP, equilibrated with 10 $\mu M$ fumarate, was mixed with 2 mM L-malate. Fitting the trace to a single exponential function indicated a rate of 11.7 s$^{-1}$. The lower record (B) shows the decrease in protein fluorescence when 4 $\mu M$ DctP was mixed with 200 $\mu M$ fumarate. Fitting the data to a single exponential function indicated a rate of 8.4 s$^{-1}$. The standard errors of the fitted rates are less than 10%.](image)

![Fig. 7. Comparison of the stopped-flow records showing 200 $\mu M$ fumarate binding to 4 $\mu M$ native DctP (upper trace, A) and 4 $\mu M$ urea treated DctP (lower trace, B). The native protein was denatured with 4 x urea to remove any endogenously bound ligand and then renatured by dialysis into tris buffer, as described under "Materials and Methods." Analyzing the two traces by curve fitting to a single exponential with a linear drift indicated almost identical rates of 6.7 and 7.0 s$^{-1}$, respectively. The standard errors of the fitted rates are less than 10%.](image)
The above studies clearly demonstrate that the renatured and native protein are kinetically equivalent and that the kinetics of fumarate binding are inconsistent with the protein having any endogenously bound ligand following purification. In view of the fact that we have failed to find any evidence for any residual ligand binding, which in any event would have been removed by the urea treatment, we conclude that the protein must be undergoing a slow conformational change. Accordingly, we have analyzed our kinetic data in terms of Scheme I.

Fitting the data to the equation governing Scheme I gave values for rate constants \( k_1 \) and \( k_{-1} \) of 6.1 (±0.90) s\(^{-1}\) and 174 (±37.5) s\(^{-1}\), respectively, and an equilibrium constant \( (K_5) \) for fumarate binding of 0.12 (±0.036) μM. The overall equilibrium constant will be higher than \( K_5 \) due to the partitioning between the two conformational forms,

\[
K_5 = K_2 \left( 1 + \frac{K_1}{K_2} \right)
\]

(6)

where \( K_5 \) is the overall dissociation constant, \( K_2 \) the dissociation constant for the BP1-fumarate complex, and \( K_1 \) is the equilibrium constant for the BP1 to BP2 transition \( (K_2 = k_6/k_{-5}) \). With an equilibrium constant of 0.035 for \( K_2 \), this would indicate that the overall dissociation constant should be about 3.5 μM. This value is considerably higher than that obtained from the steady state fluorescence titration data for fumarate \( (K_5 = 0.255 \text{ μM}) \) and would suggest that there is a further, optically silent, isomerization of the protein following fumarate binding.

\[
\begin{align*}
\text{BP}1 & \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} [L] \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} \text{BP}_2-L \overset{k_3}{\underset{k_{-3}}{\rightleftharpoons}} \text{BP}_3-L
\end{align*}
\]

SCHEME III

Assuming such a model, the equilibrium constant for step 3, \( K_3 \), can be calculated from the overall affinity constant, determined from the fumarate fluorescence titration data, and the equilibrium constants for steps 1 and 2, determined from our analysis of the binding kinetics.

\[
K_3 = \frac{K_5 (1 + K_1)}{K_1 K_2} - 1
\]

(7)

\( K_5 \) is the overall affinity constant \( (K_5 = 1/K_2 \) from Table I), \( K_1 \) is the equilibrium constant for the isomerization between the BP1 and BP2 forms \( (K_1 = k_6/k_{-5}) \), and \( K_2 \) the affinity constant for ligand binding to the BP2 form \( (K_2 = k_6/k_{-5}) \).

Using the values quoted in Tables I and III a value of 12.9 was calculated for \( K_3 \) (see Table V). In view of the fact that the time-resolved fumarate binding data readily fits Equation 4, this would suggest that step 2 is a rapid equilibrium process. Making this assumption, \( k_3 \) will be the measured dissociation rate constant in ligand displacement experiments. \( k_3 \) can then be simply calculated from the equilibrium constant for step 3.

\[
k_3 = k_{-3} \cdot K_3
\]

(8)

Consistent with this interpretation, both the fumarate binding and dissociation time courses appear to be single exponential processes. Accordingly, \( k_3 \) was calculated to be 151 s\(^{-1}\) (see Table V).

The Binding of Alternative Substrates—Although small, the signal change associated with L-malate, D-malate, and succinate binding were sufficient to allow us to time-resolve the conformational change associated with the binding of these ligands at near saturating ligand concentrations. As illustrated in Fig. 8, they were all found to bind with similar rates. This is consistent with a ligand induced isomerization of the protein, as shown in Scheme I. Furthermore, identical kinetic behavior was observed for the binding of these ligands to urea treated protein.

Over the concentration range for which a signal change for L-malate binding could be observed (12 μM protein mixed with 1–2000 μM L-malate), the rate remained constant at a value of about 10–11 s\(^{-1}\). This behavior is probably due to the high affinity binding of L-malate, a conclusion that is highly consistent with our steady state measurements of L-malate binding, which indicate a \( K_5 \) for L-malate of about 0.05 μM. Consequently, it has not been possible to determine the affinity of the BP2 conformation for L-malate.

D-Malate, on the other hand, has a much lower affinity than L-malate and data could be accurately obtained at concentrations both above and below the apparent \( K_5 \) (Fig. 9). In accord with Scheme I, the apparent binding rate was found to decrease hyperbolically with increasing ligand concentration. Fitting the data from this plot to Equation 1 indicated that the apparent binding rate decreased with similar \( k_1 \) and \( k_{-1} \) values but with a different \( K_5 \) to that obtained for fumarate (Table III). This kinetic behavior is consistent with the model given by Scheme I, in which the values for \( k_1 \) and \( k_{-1} \) are independent of which ligand is binding to the protein.

Succinate produces about a 2-fold greater enhancement in fluorescence than L- or D-malate. This, coupled with the fact that it has about a 3–5-fold lower affinity than L-malate, has...
These were calculated using the relationship, where the rates rather than from differences in the dissociation rates. 

TABLE III

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Kinetic parameters</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$k_1$</td>
<td>$k_{-1}$</td>
<td>$K_d$</td>
</tr>
<tr>
<td>Fumarate</td>
<td>$6.1 \pm 0.90$</td>
<td>$174 \pm 37.5$</td>
<td>$0.12 \pm 0.036$</td>
</tr>
<tr>
<td>L-Malate$^*$</td>
<td>$6.0 \pm 0.58$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Malate</td>
<td>$11.1 \pm 3.14$</td>
<td>$167 \pm 5.3$</td>
<td>$25.5 \pm 3.18$</td>
</tr>
<tr>
<td>Succinate</td>
<td>$9.4 \pm 0.97$</td>
<td>$173 \pm 11.5$</td>
<td>$0.26 \pm 0.021$</td>
</tr>
</tbody>
</table>

*The $k_1$ value quoted for L-malate was obtained from a single exponential (with sloping base line) fit of the stopped-flow record for 200 μM L-malate binding to 12 μM DctP. $k_1$ and $K_d$ could not be determined for L-malate due to the small signal change upon its binding.

TABLE IV

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$k_{appr}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fumarate</td>
<td>$11.7 \pm 0.08$</td>
</tr>
<tr>
<td>L-Malate</td>
<td>$9.3 \pm 0.04$</td>
</tr>
<tr>
<td>D-Malate</td>
<td>$7.9 \pm 0.04$</td>
</tr>
<tr>
<td>Succinate</td>
<td>$9.1 \pm 0.06$</td>
</tr>
</tbody>
</table>

allowed the determination of its apparent binding rate for a series of concentrations. This has enabled us to fit the data to Scheme I and to establish that here too, $k_1$ and $k_{-1}$ have similar values to those obtained for fumarate.

The dissociation rate constants for L-malate, D-malate, and succinate were measured independently by displacement with fumarate, and are tabulated in Table IV. In the case of D-malate and succinate the values obtained are clearly inconsistent with ligand binding being rate-limited by the dissociation of endogenous ligand, since in both cases the predicted dissociation rate constants should have been in excess of 100 s$^{-1}$. The small change in the dissociation constants would indicate that the differences in affinity of the four ligands is attributable to differences in the apparent association rates. These were calculated using the relationship,

$$k_{app} = k_{appr}/K_d$$

where $k_{app}$ is the apparent association rate for ligand binding to BP$_2$, $k_{appr}$ is the measured dissociation rate constant, and $K_d$ is the dissociation constant for the BP$_2$:L complex. The values tabulated in Table V clearly establish that the differing affinities result from the differences in the apparent association rates rather than from differences in the dissociation rates.

FIG. 9. The (L-malate) dependence of the apparent binding rate. The curve through the data points represents the best fit of the data according to Equation 4. The quoted concentrations are those in the mixing chamber of the stopped-flow spectrometer.

Calculation of the predicted overall dissociation constants according to Equation 6 for D-malate and succinate gives values that are significantly higher than those obtained from steady state titrations. This again would indicate that there is an additional isomerization of the protein following ligand binding, as proposed for fumarate binding. The values calculated for $K_a$ and $K_b$, using Equations 7 and 8, are tabulated in Table V.

DISCUSSION

The fluorescence properties of the C4-dicarboxylate binding protein from *Rb. capsulatus* have been determined. Fumarate binding quenches the fluorescence of 4 μM protein by about 17%, whereas the other ligands tested all induce a small enhancement in the fluorescence, with succinate producing a slightly larger increase than L- or D-malate. The contrast in the fluorescence change for fumarate with that for the other three ligands might suggest that fumarate supports a different conformational form of the protein. This supposition was tested by KI titration of the protein fluorescence. Potassium iodide is a hydrophilic collisional quencher of tryptophan residues and can be used to probe their accessibility. As such, it can be used to test for a putative conformational change by detecting whether the tryptophans are in different environments in the alternate conformations. For the ligand-free protein, 37% of the tryptophans were found to be accessible. Although this might suggest that 1 of the 3 tryptophans present in DctP is located near the surface of the protein, whereas the other two are buried within the protein, we must consider that greater than one-third of the tryptophans are apparently exposed. Two possible interpretations of this finding might be that (a) two or more of the tryptophans are partially accessible to the quencher or (b) that there are two completely accessible tryptophans, but one of these is only exposed in about 10% of the protein population. This latter proposition could be explained in terms of a conformational equilibrium in which the protein oscillated between two conformations in which the tryptophan residue was alternately exposed and shielded from the aqueous medium. This proposition would be consistent with the preceding analysis of the kinetics of ligand binding, in which we have deduced that the protein alternates between two conformational forms, which differ in their ability to bind ligand. Under our assay conditions, around 96.5% of the protein was found to be in the non-ligand-binding form, but the increase in ionic strength due to KI addition might push the equilibrium towards the binding form. It is tempting to suggest that these two forms represent the closed- and open-unliganded forms and that one of the tryptophans involved resides within the ligand binding site. Fumarate was found to decrease the proportion of accessible tryptophans to 31%, with little change in the quenching constant. Since the quenching constant can be taken as a crude measure of the accessibility of the tryptophans in the protein, this would indicate that fumarate is not changing the local environment of the quenchable residues but merely reducing their proportion. Again this would be consistent with fumarate causing a tryptophan to be shielded from the aqueous environment, possibly by stabilizing the closed conformation. This reduction cannot simply be attributed to fumarate directly shielding a tryptophan residue in the binding site with no conformational change for two reasons. First, fumarate binding causes a relatively large quench in tryptophan fluorescence, which would be expected to be reflected by an equivalent reduction in KI quenching. Second, one would expect to see a reduction in KI quenching for L-malate, but in fact this ligand increases the proportion
of accessible tryptophans to 42%. Thus, we propose that there are 2 tryptophans which are equally accessible to quenching in the open conformation. Possibly, one is located close to the binding site and the other on the surface of the protein, with the 3rd residue buried within the protein. KI can quench the fluorescence of the surface residue in either conformation, but the binding site residue can only be quenched when the protein is in the open conformation. Fumarate quenches the fluorescence of the binding site residue, but because only about 5–10% of the protein is in the open conformation prior to ligand binding, this is reflected by a slight drop in KI quenching. However, the L-malate data would suggest that fumarate must induce a conformational change which causes the fluorescence of the putative binding site tryptophan to be quenched. Otherwise L-malate, which can also sit in the binding site, would presumably cause a quench in the fluorescence. L-Malate causes a slight increase in the number and accessibility of the tryptophans, suggesting that it supports a different conformation of the protein to fumarate and one in which the tryptophans are in a more aqueous-exposed environment.

By using stopped-flow fluorescence spectroscopy it was possible to time-resolve both the ligand association and dissociation processes. The observed kinetic behavior was found to be consistent with a slow isomerization between two different conformational states of the binding protein, with only one of these conformational states able to bind ligands. Ligand binding appears to be a rapid equilibrium process, which induces a further conformational change in the protein. X-ray crystallographic studies on other binding proteins have revealed that these proteins can adopt at least three different conformational states: the open-unliganded state, the open-liganded state, and the closed-liganded state (Sack et al., 1989a, 1989b; Quiocho, 1990). Together, these studies have suggested a model for the operation of periplasmic binding proteins which the tryptophan accessibility of the tryptophans, suggesting that it supports a different conformation of the protein to fumarate and one in which the tryptophans are in a more aqueous-exposed environment.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Equilibrium</th>
<th>Apparent association rate k_{on}/K_{d}</th>
<th>k_{d}/k_{o}</th>
<th>K_{d}</th>
<th>k_{o}</th>
<th>k_{d} (k_{on})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_{d}</td>
<td>k_{i}</td>
<td>k_{-}</td>
<td>K_{d}</td>
<td>k_{i}</td>
<td>k_{-} (k_{on})</td>
</tr>
<tr>
<td>Fumarate</td>
<td>0.255</td>
<td>6.1</td>
<td>174</td>
<td>0.12</td>
<td>0.01</td>
<td>0.006</td>
</tr>
<tr>
<td>L-Malate</td>
<td>0.05</td>
<td>6.0</td>
<td>&gt;0.05</td>
<td>&lt;1.0</td>
<td>&lt;0.01</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>D-Malate</td>
<td>6.3</td>
<td>11.1</td>
<td>167</td>
<td>25.5</td>
<td>0.31</td>
<td>1.7</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.17</td>
<td>9.4</td>
<td>173</td>
<td>0.26</td>
<td>36.0</td>
<td>3.1</td>
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</tbody>
</table>

a An upper limit for the L-malate K_{d} and k_{o} values was obtained by using the fumarate values for K_{d} and k_{o}.

have shifted any equilibrium between conformations and allowed the transition to be detected. One piece of evidence, however, might support the suggestion of a conformational equilibrium in the absence of ligand. Parsons and Hogg (1974) have reported that the affinity of the arabinose binding protein increases with pH, with a pK_{a} of around 4.3. They have correlated this behavior with a decrease in the fluorescence of the protein at pH values below the pK_{a}, suggesting that at low pH the protein adopts a non-ligand-binding conformation. The ligand binding affinity of the DctP protein decreases in the order L-malate > succinate > fumarate > D-malate. In spanning this ligand series the binding constant decreases by at least 2 orders of magnitude. This decrease in affinity is clearly attributable to a decrease in the apparent association rate constants, rather than in the dissociation rate constants, which were found to be of similar magnitude (Table V). This contrasts with several sugar and amino acid binding proteins (Miller et al., 1983) where differences in affinity were found to be due to variation in the dissociation rate constants alone. For DctP, L-malate has the highest apparent association rate constant, which was estimated to be (at most) 1.86 × 10^{8} M^{-1} s^{-1}, whereas D-malate had the lowest, with a rate of 3.1 × 10^{6} M^{-1} s^{-1} (see Table V). The value for D-malate is 3–4 orders of magnitude lower than expected for a diffusion controlled process and would strongly support the suggestion that ligand binding to the BP_{2} conformation is a two-step process. The data for fumarate and succinate binding would similarly support this conclusion. It appears that the forward rate constant for formation of the BP_{2}L complex is the affinity determining step for L-malate, succinate, and fumarate. In the case of D-malate, k_{o} was found to be comparable with that determined for L-malate. This indicates that the substantial difference in equilibrium K_{d} values and the K_{d} values obtained for the BP_{2}L complexes for L- and D-malate, cannot be attributed to k_{o}. The data suggest that this difference resides with step two, the initial encounter of ligand and protein. One possible explanation of this difference might be that D-malate cannot freely enter the DctP substrate binding site. Furthermore, there does not appear to be a strict stereochemical requirement for a C2 hydroxyl in either the L- or D-configuration, since succinate is also bound with relatively high affinity. This again would suggest that the C2 hydroxyl of D-malate sterically hinders binding. Indeed, little difference was found in the dissociation rate constants for L- and D-malate, suggesting that no additional bonds are involved in holding the L-isomer. The high affinity of the protein for fumarate suggests that the double bond which constrains the two carboxyl groups in the trans configuration is not detrimental for ligand binding. We have shown previously that maleate, in which the two carboxyls are constrained in the cis position by the double bond, has a much lower affinity (Shaw et al., 1991). This would suggest that the trans carboxyl configuration is the preferred orientation of these functional groups for binding. In accord with our previous study (Shaw et al., 1991),
pyruvate did not appear to bind to DctP, indicating a requirement for two carboxyl groups.

REFERENCES
The mechanism of ligand binding to the periplasmic C4-dicarboxylate binding protein (DctP) from Rhodobacter capsulatus.

A R Walmsley, J G Shaw and D J Kelly


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