Perturbation of the Equilibrium between Open and Closed Conformations of the Periplasmic C4-Dicarboxylate Binding Protein from *Rhodobacter capsulatus*†

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ABSTRACT: A kinetic and thermodynamic analysis has been carried out on the conformational transitions of the periplasmic C4–dicarboxylate binding protein (DctP) from the photosynthetic bacterium *Rhodobacter capsulatus*. This protein is distinct from other periplasmic binding proteins characterized to date in that the transition between the putative closed-unliganded (BP_1) and open-unliganded (BP_2) conformations is slow compared to the rate of ligand binding [Walmsley, A. R., Shaw, J. G., & Kelly, D. J. (1992) *J. Biol. Chem. 276*, 8064–8072]. Using stopped-flow fluorescence techniques, we have probed the conformational dynamics of the closed to open transition of DctP in the absence and presence of ligand. Both the forward rate constant for the BP_1 to BP_2 interconversion (k_1) and the fumarate dissociation rate constant (k_{-3}) were found to increase in a biphasic manner between pH 5 and pH 11. The data were fitted to a two-pK_a function which gave pK_a values of 10.3 and 5.4 for the BP_1 to BP_2 interconversion and 8.9 and 4.5 for the closed-liganded (BP_3L) to open-liganded (BP_2L) transition. An increase in ionic strength at constant pH resulted in a hyperbolic increase in both k_1 and k_{-3} to maximal rates that were similar in each case to the values obtained in pH variation experiments. Measurement of the temperature dependencies of k_1 and k_{-3} also gave similar activation energies. Gibbs free energy, enthalpy, and entropy changes were determined for the open to closed transitions of DctP in both the presence and absence of ligand. All the data were found to be consistent with the existence of a salt bridge between an acidic and a basic residue in the protein which is involved in stabilizing the closed conformation.

Periplasmic binding proteins are an integral component of an important and widespread class of bacterial solute transport system (Ames, 1988; Quiocho, 1990). High-resolution structures determined by X-ray crystallography are now available for several binding proteins (Quiocho & Vyas, 1984; Pflugrath & Quiocho, 1985, 1988; Vyas et al., 1987; Sack et al., 1989a,b; Leucke & Quiocho, 1990; Quiocho, 1990) and have revealed that each protein consists essentially of two folded globular domains connected by a hinge region. Ligand binding occurs between the two domains, which close around the ligand during its sequestration. This behavior can be modeled by Scheme I where L is the ligand, BP_1 and BP_2 represent the closed-unliganded and open-unliganded conformations of the protein, respectively, and BP_2L and BP_3L represent the open-liganded and closed-liganded conformations, respectively. Structural evidence for the existence of the BP_2, BP_2L, and BP_3L forms in a variety of binding proteins is strong (Sack et al., 1989a,b; Quiocho & Vyas, 1984; Pflugrath & Quiocho, 1988) while that for the BP_1 form is weaker, although there are reports that the arabinose binding protein can adopt this conformation [see Newcomer et al. (1981) and Vermersch et al. (1990)]. A recent NMR study of the galactose binding protein (Luck & Falke, 1991) also supports this supposition.

Direct kinetic evidence to support all of the steps in Scheme I has been difficult to obtain, particularly for the BP_1 and BP_2 transition. Most studies of ligand binding have revealed that the association rate has a positive linear dependence upon the ligand concentration, indicative of a simple one-step binding process (Miller et al., 1980, 1983). These data are not inconsistent with the model, but would indicate that the transition between closed and open conformations is a rapid equilibrium process. More recently, Walmsley et al. (1992) have shown that for the C4-dicarboxylate binding protein (DctP) purified from *Rhodobacter capsulatus* (Shaw et al., 1991), this situation is reversed; the transition between open-unliganded and closed-unliganded conformations is slow relative to ligand binding. In this case, the transition between conformations was sufficiently slow to allow its direct measurement by stopped-flow fluorescence spectroscopy, providing the first substantive kinetic evidence for all of the steps in the model of Scheme I. Little is known of the molecular dynamics of the conformational change between the open and closed conformations in periplasmic binding proteins. One recent investigation by Jacobson et al. (1992) has revealed that two salt bridges, which span the ligand binding cleft of the sulfate binding protein, are involved in stabilizing the closed conformation.

Scheme I

$$BP_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} BP_2 \underset{k_{-2}}{\overset{k_2[L]}{\rightleftharpoons}} BP_2L \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} BP_3L$$

In the present investigation, we have probed the conformational dynamics of the closed to open transition of DctP, in the presence and absence of bound ligand, by altering the pH, temperature, and ionic strength of the medium. All three conditions were found to perturb the conformational equilibrium, and evidence was obtained which indicated that a salt bridge was involved in stabilizing the closed conformation.

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EXPERIMENTAL PROCEDURES

Media and Growth Conditions. Rhodobacter capsulatus strain 37b4 (wild type) was used throughout this work. Bacteria were grown under aerobic conditions in the dark at 30 °C on a rotary shaker in minimal RCV medium (Weaver et al., 1975) with 30 mM DL-malate as the carbon source. Cells were harvested at the end of the exponential phase by centrifugation.

Purification of the C4-Dicarboxylate Binding Protein. The DctP protein was isolated by ammonium sulfate fractionation of periplasmic extracts followed by FPLC ion-exchange chromatography as described previously (Shaw et al., 1991). The purity of the protein was assessed by SDS–PAGE on 12.5% gels. Only a single band of molecular weight 33 000 was obtained after Coomassie blue staining.

Assay Conditions. Unless stated otherwise, experiments were performed in a composite buffer mixture of 10 mM 2-amino-2-methyl-1-propanol hydrochloride, 10 mM Trizma, and 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) at the required pH. This buffer covers the pH range 5–11. The standard temperature was 20 °C, except in temperature variation studies.

Ligand Binding Assay. The dicarboxylate binding capacity of DctP was routinely checked for consistency between different preparations, according to the following procedure. [^{14}C]-L-Malate (1.85–2.2 MBq mmol^{-1}) was added to a solution of DctP (1.0 mg mL^{-1}) in 10 mM Tris-HCl buffer, pH 8.0, to a final concentration of 8 μM and incubated for 10 min on ice. The protein was then precipitated by the addition of 1 mL of cold saturated ammonium sulfate solution. The protein was filtered (0.45-μm nitrocellulose filter disks) and washed with 4 mL of cold saturated ammonium sulfate, and then the filters were added to 3 mL of scintillation fluid and counted.

Spectroscopic Equipment. Rapid reactions were followed using an Applied-Photophysics (London, United Kingdom) stopped-flow spectrofluorometer, routinely operated at 20 °C. Tryptophan fluorescence was excited at 297 nm, selected by focusing a 150-W Xenon arc lamp onto an f/3.4 monochromator, and the emitted light was selected with a WG335 Schott filter, positioned in front of the observation PMT. Data were captured on an Archimedes 420/1 microcomputer, and analyzed by nonlinear regression. Generally, data acquisition was carried out over a split time-base, with the first half of the data captured over one-fifth as short a time as for the second half. In this manner, data were collected both during the initial exponential phase and during the latter linear phase, when the fluorescence signal had approached its steady-state level. This procedure allows for more accurate determination of the rate and amplitude of an exponentially changing signal (Walmsley & Bagshaw, 1989). The data were electronically filtered using a filter time constant at least one-tenth that of the half-time of the recorded trace and in most cases one-hundredth the half-time. At least three stopped-flow traces were averaged prior to nonlinear regression analysis. Steady-state fluorescence data were acquired on a Jasco FP-777 spectrofluorometer, routinely operated at 20 °C.

Measurement of Conformational Transition Rates. Ligand binding to DctP from *Rhodobacter capsulatus* can be described in terms of the four-step mechanism described by Scheme I (Walmsley et al., 1992). The dynamics of the individual steps were investigated by the following procedures. The first step, which is thought to be an isomerization between closed and open conformations (e.g., nonbinding and binding forms of the protein), was investigated by mixing DctP with fumarate in the stopped-flow spectrometer. The resulting decrease in protein fluorescence, due to DctP–fumarate complex formation, was monitored. The rate constant for BP_1 to BP_2 interconversion was determined by mixing the protein with a saturating concentration of fumarate. We have shown previously that DctP has a submicromolar K_d for fumarate (Walmsley et al., 1992). Accordingly, 100 μM fumarate was used to draw DctP (2 μM) into the BP_2 conformation. k_{-1} and $1/K_2$ were obtained from a hyperbolic fit of the ligand concentration dependence of the apparent binding rate, which decreases hyperbolically (Walmsley et al., 1992). Conversely, if the protein (4 μM DctP) is saturated with fumarate (10 μM) and then mixed with 2 mM succinate (an alternative high-affinity ligand for the protein), a transient increase in protein fluorescence can be observed. This is attributable to fumarate dissociation, since succinate produces a small increase and fumarate a moderate decrease in protein fluorescence (Walmsley et al., 1992). The high concentration of succinate employed in these experiments ensured that succinate binding was rapid and that the reaction was rate-limited by fumarate dissociation. The rate constants for these processes were determined as a function of pH, temperature, and ionic strength.

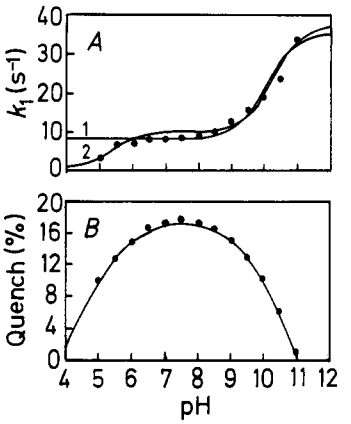
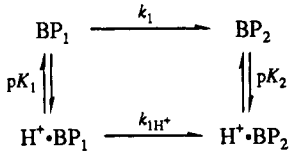


FIGURE 1: pH dependence of the rate constant and signal amplitude for the transition from the closed to the open conformation of DctP (k_1). k_1 was measured over a range of pH values by mixing 4 μM DctP with 200 μM fumarate in the stopped-flow device, and monitoring the decrease in protein fluorescence due to ligand binding (excitation 297 nm, emission >335 nm). Both the protein and fumarate solutions were adjusted to the required pH. (A) Variation of k_1 as a function of pH at 20 °C. Curve 1 is the nonlinear least-squares regression best fit to a single-pK_a function. Curve 2 is the best fit to a double-pK_a function (see eq 1 under Results). (B) Variation in the signal amplitude as a function of the pH. The curve through the data points represent the best fit of the data to a double-pK_a function (eq 2 under Results).

RESULTS

pH Dependence of the *BP_1–BP_2 Conformational Change.* The rate constants for BP_1 to BP_2 interconversion were determined as a function of pH by mixing the protein (4 μM) with a saturating concentration of fumarate (200 μM), with both components buffered to the required pH. Data were collected between pH 5.0 and 11, with lower and higher pHs being unobtainable due to denaturation of the protein. The rate of conformational change between BP_1 and BP_2 forms was found to be pH-dependent, with an increasing rate that could be described by a single pK_a of 10.0 (±0.18), the rate constant varying between 7.8 (±0.84) and 34.4 (±3.00) s^{-1} in going from low to high pH (Figure 1A). This pK_a is well removed from the pK_a's of fumarate (pK_a1 = 3.03, pK_a2 = 4.44), indicating that this represents the pK_a for a group on

Scheme II



the protein. However, attempts to fit the data to a single rate constant which varied according to the pK_a were unsuccessful. Particularly at low pHs there was a systematic deviation of the data from the best-fit curve, indicating that the data followed a more complex pH function than that described by a single-pK_a model.

These results can be interpreted in terms of a pH-dependent equilibrium between the BP_1 and BP_2 conformations. The fact that the rate does not extrapolate to zero at low pH (Figure 1) would indicate that both the protonated and deprotonated forms of BP_1 and BP_2 can undergo interconversion. The first step of Scheme I can be adapted to include these pH effects as shown in Scheme II. Accordingly, the data were analyzed in terms of a two-pK_a model for Scheme II, which would be consistent with the continuous increase in rate across the pH range studied:

$$k_{obs} = \frac{k_1}{1 + 10^{pK_1-pH}} + \frac{k_{1H^+}}{1 + 10^{pK_2-pH}} \quad (1)$$

Fitting the data to this equation gave values for the constants of k_1 = 27.5 (±2.42) s^{-1}, pK_1 or pK_2 = 10.3 (±0.13), k_{1H^+} = 9.6 (±0.67) s^{-1}, and pK_2 or pK_1 = 5.4 (±0.21). Although it was not possible to determine which pK_a value was associated with a particular conformational form, the large difference in the pK_a's for the BP_1 and BP_2 forms would suggest that these pK_a's refer to two different groups on the protein, rather than the same group having different pK_a's in the two conformations (see Figure 1A).

Better resolution of the two pK_a's was found to be possible from an analysis of the corresponding signal amplitude changes for saturating fumarate binding at different pHs. The variation in the signal amplitude (protein fluorescence quench) as a function of the pH yields the bell-shaped curve shown in Figure 1B. An equivalent bell-shaped curve was obtained when the steady-state fluorescence of the DctP–fumarate complex was determined as a function of the pH (data not shown). The amplitude data were fitted to the following two-pK_a function for variation of the protein quench (ΔF) between zero and the maximal level (F_t):

$$\Delta F = \frac{F_t}{1 + 10^{pK_1-pH} + 1 + 10^{pH-pK_2}} \quad (2)$$

This yielded values for the constants of F_t = 17.3 (±0.17), pK_1 or pK_2 = 5.1 (±0.04), and pK_2 or pK_1 = 10.1 (±0.06). Again, it seems reasonable to conclude that the lower value pK_a is for a group on the protein, rather than for fumarate, since it is significantly higher than the known pK_a values for fumarate. Moreover, the pH would have to be dropped by at least 2 units below the pK_a for fumarate before the concentration of deprotonated fumarate, the binding species, becomes nonsaturating under the prevailing experimental conditions (2 μM DctP and 100 μM fumarate).

To test whether only the forward rate constant for the conformational change (k_1) was pH-dependent, or whether the reverse rate constant (k_{-1}) or the apparent affinity of the protein for fumarate ($1/K_2$) were also pH-dependent, fumarate binding was investigated at pH 6, 8, and 10.5. The fumarate concentration dependence of the apparent binding rate was determined for each pH. As we have shown previously, the hyperbolic decrease in the rate for the observed transient can be fitted to the equation (Walmsley et al., 1992):

$$k_{obs} = k_1 + k_{-1}/(1 + [L]K_2) \quad (3)$$

The fitted parameters for the data obtained at these pHs are given in Table I. At pH 6.0, there is a decrease in both k_1 and k_{-1}, with a slight shift in the equilibrium toward the closed conformation (e.g., 93.9% of the DctP is in the closed conformation at pH 6, compared with 92.8% at pH 8). In contrast, the apparent dissociation constant ($1/K_2$) increases about 7-fold from 0.05 μM at pH 8.0 to 0.37 μM at pH 6.0. Toward the other pH extreme (pH 10.5), k_1 increases, while k_{-1} decreases. These changes result in a large shift in the equilibrium toward the BP_2 form, with around 41% of the binding protein molecules adopting the open conformation. However, this shift in equilibrium occurs concomitantly with a decrease in the fumarate binding affinity of the protein, so that the overall affinity of the protein is not substantially changed at this pH. The data also indicate that k_{-1} follows a two-pK_a function, with this rate constant being lower at both pH 6 and 10.5, relative to pH 8.0.

The shift in equilibrium upon increasing the pH above 8 ought to be ameanable to direct measurement. To test this prediction, the protein (4 μM in 1 mM Tris, pH 8) was pH-jumped from pH 8 to 10 by mixing with an equal volume of a 30 mM composite buffer mixture in the stopped-flow device. A transient decrease in protein fluorescence could be observed under such conditions (Figure 2, trace a). The amplitude of the signal change, about a 4% quench in fluorescence, is reasonably consistent with the expected shift in equilibrium. However, the rate of signal change was more than twice that which characterized saturating fumarate binding at pH 10 (compare traces a and b in Figure 2). This behavior may be attributable to the fact that during such experiments the protein is simultaneously undergoing a jump in both pH and ionic strength. Indeed, we have found that increasing the buffer concentration from 10 mM to 30 mM causes about a 50% increase in k_1 at pH 8. Preequilibrating the protein (4 μM,

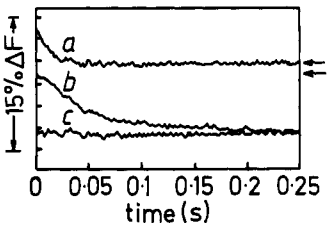


FIGURE 2: Change in protein fluorescence when the pH of the medium is jumped from 8.0 to 10.0. The upper trace (a) shows the decrease in protein fluorescence when the protein (4 μM DctP in 1 mM buffer, at pH 8.0) was mixed with 30 mM buffer at pH 10.0. The central trace (b) is for fumarate binding at pH 10.0 (in 30 mM buffer). The arrows indicate the extent of the initial rapid quench in fluorescence which occurs at a rate beyond the resolution of the stopped-flow device used here. The lower trace (c) was obtained by preequilibrating the protein (4 μM DctP in 1 mM Tris, pH 8) with 100 μM fumarate prior to pH-jumping the protein with 100 μM fumarate in 30 mM buffer, pH 10.

Table I: Kinetic Parameters for Fumarate Binding to DctP at Different pHs

pH	k_1 (s^{-1})	k_{-1} (s^{-1})	$1/K_2$ (μM)
6.0	6.0 ± 2.49	83.2 ± 12.8	0.37 ± 0.133
8.0	9.90 ± 0.85	161.0 ± 25.3	0.05 ± 0.017
10.5	32.2 ± 4.63	78.3 ± 22.8	0.57 ± 0.410

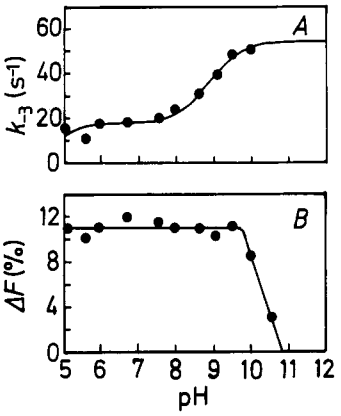


FIGURE 3: pH dependence of the rate constant and signal amplitude for the transition from the closed to the open conformation of DctP in the presence of bound ligand (k_{-3}). (A) pH dependence of k_{-3}. The curve through the data points represents the best fit to a double-pK_a function (eq 1 under Results). (B) pH dependence of the signal amplitude, plotted as the increase in fluorescence upon mixing fumarate-loaded DctP with excess succinate.

in 1 mM Tris, pH 8) with 100 μM fumarate prior to pH-jumping the protein with 100 μM fumarate (in 30 mM buffer, pH 10) abolishes the signal change (Figure 2, trace c) as would be expected if the fumarate sequesters the protein in the BP_2–fumarate form. Also consistent with the interpretation that an increased proportion of the protein is in the BP_2 form at pH 10 is the finding that the initial rapid quench in fluorescence [which occurs at a rate which is beyond the time-resolution limit of the stopped-flow device; see Walmsley et al. (1992)] increased to around 15% of the total quench amplitude at this high pH (Figure 2, arrows).

The drop in fluorescence quenching at pHs above and below 8 can be reconciled with a shift in the equilibrium between the two conformations. If it is assumed that pK_a1 = 10.3, then at pH 8 the rate constants for interconversion of the two conformations indicate that 95.3% of the protein molecules are in the BP_1 form, which will largely be protonated (see Table I). Conversely, the small fraction of protein molecules in the BP_2 form will be deprotonated. As the pH is increased from pH 8, there will be a shift in the equilibria toward the deprotonated BP_1 form, which interconverts more readily to the BP_2 form, consequently pushing the equilibrium toward the BP_2 form. On the other hand, decreasing the pH below pH 8 would lead to protonation of the BP_2 form, pulling the equilibrium toward this conformation. With the fluorescence quench associated with fumarate binding largely attributable to the interconversion of BP_1 to BP_2, the fluroescence signal will decrease with any shift in pH around pH 8. The BP_1 concentration can be viewed as maximally poised around the midpoint of the two pK_a's.

The fumarate dissociation rate constant was determined as a function of the pH by preequilibrating the protein (4 μM in buffer at the desired pH) with 10 μM fumarate, which was then displaced with 2 mM succinate (buffered to the desired pH) in the stopped-flow spectrometer (Figure 3a). The dissociation rate constant (k_{-3}) was found to have a similar pH dependence to that for isomerization between the closed and open conformations (k_1) and was readily fitted to the two-pK_a function given above (eq 1). This analysis yielded values for the constants of k_1 36.7 (±5.04) s^{-1}, pK_1 or pK_2 = 8.9 (±0.20), k_{1H^+} = 17.6 (±1.50) s^{-1}, and pK_2 or pK_1 = 4.5 (±0.41). In contrast to the data for k_1, we found that the signal amplitude did not change substantially with the pH (Figure 3B), except for a decrease at very high pH which was

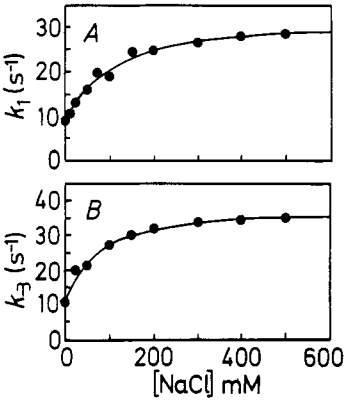


FIGURE 4: Ionic strength dependence of k_1 and k_{-3}. The rate constants k_1 and k_{-3} are plotted as a function of [NaCl] in (A) and (B), respectively. The quoted salt concentrations are additional to the standard 30 mM buffer (pH 8.0, 20 °C) used in all assays. The data were fitted to a hyperbolic function in order to ascertain the maximal rate for each rate constant.

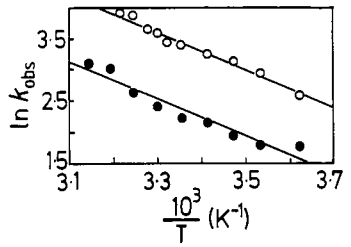


FIGURE 5: Arrhenius plots of the temperature dependencies of k_1 (●) and k_{-3} (○). The activation energies for k_1 and k_{-3} were calculated by a direct fit of the data to the Arrhenius equation by nonlinear least-squares regression. This analysis yielded activation energies of 29.8 (±2.70) kJ mol^{-1} and 27.6 (±0.33) kJ mol^{-1} for k_1 and k_{-3}, respectively.

probably due to denaturation. This finding is consistent with 10 μM fumarate being sufficient to hold nearly all of the DctP in the BP_3–L conformation at each pH. Displacement of the fumarate with succinate simply changes the ligand bound to the BP_3 conformation without affecting its concentration.

Ionic Strength Effects upon k_1 and k_{-3}. The effect of increasing ionic strength upon k_1 and k_{-3} is illustrated in Figure 4. Both rate constants were found to increase hyperbolically with the NaCl concentration, reaching a maximal level at around 0.5 M. In both cases, the maximal rate obtained by fitting the initial data to a hyperbolic function was similar to the maximal rate obtained in pH variation studies. This would suggest that increasing ionic strength destabilizes the closed conformation, rather than some nonspecific effect upon the protein. At higher concentrations, there is a rapid decrease in these rate constants, which may be attributable to protein denaturation, as observed at extreme pHs.

Temperature Dependence of the Conformational Change. The Arrhenius diagrams in Figure 5 show that both k_1 and k_{-3} have linear logarithmic temperature dependencies. This finding is consistent with our assumption that under the prevailing experimental conditions used for determining k_1 and k_{-3}, we are in fact measuring single rate constants. The activation energies for these two steps, determined by fitting the kinetic data directly to the Arrhenius equation, were found to have similar values: 29.8 (±2.70) kJ mol^{-1} and 27.6 (±0.33) kJ mol^{-1} for k_1 and k_{-3}, respectively.

The concentration dependence of fumarate binding was measured at four temperatures between 5 and 20 °C, so that the temperature dependence of k_{-1} and K_2 could be determined. It was not possible to determine these parameters at higher

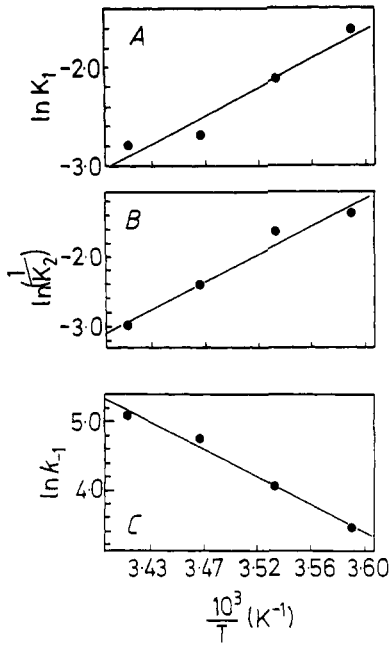


FIGURE 6: van't Hoff plot of the temperature dependence of K_1 (A) and $1/K_2$ (B). The enthalpy changes associated with conformational changes of DctP and for ligand binding were determined by linear regression. This analysis yielded enthalpies of −55 kJ mol^{-1} for the transition from the closed to open conformation of DctP and 74.7 kJ mol^{-1} for fumarate binding to DctP. (C) Arrhenius plot of the temperature dependence of k_{-1}. The activation energy of k_{-1} was calculated by linear regression analysis of the data to be 76 kJ mol^{-1}.

temperatures because the rate of the observed transient became too fast and was of insufficient amplitude for determination at low fumarate concentrations, which would be necessary to define $1/K_2$. Furthermore, our studies at low temperature have shown that $1/K_2$ increases with temperature, while the signal amplitude decreases with increasing temperature. The Arrhenius plot given in Figure 6C, for k_{-1}, indicates an activation energy of 76.0 kJ mol^{-1} for the open to closed conformational transition of the protein. The enthalpy change for this conformational change was determined from a van't Hoff plot (see Figure 6A) of the equilibrium constant, K_1, for the conformational change ($K_1 = k_1/k_{-1}$). This indicated that the closed to open transition is an exothermic process, which occurs with an enthalpy change of −55.2 kJ mol^{-1}. This value is reasonably consistent with that calculated from the difference in the activation energies for k_1 and k_{-1} ($\Delta H° = E_1 - E_{-1} = 29.8 - 76 = -46.2$ kJ mol^{-1}). The entropy change for this transition was then calculated from the relationship:

$$\Delta G° = -RT \ln K_1 = \Delta H° - T\Delta S° \quad (4)$$

This analysis yielded respective values for $\Delta G°$ and $\Delta S°$ of 6.8 kJ mol^{-1} and −212 J K^{-1} mol^{-1} at 20 °C. Similarly, the thermodynamic parameters for fumarate binding were determined from a van't Hoff plot of K_2 (see Figure 6B) and eq 4. Hence, $\Delta H°$, $\Delta G°$, and $\Delta S°$ were calculated to be 74.7 kJ mol^{-1}, −40.9 kJ mol^{-1}, and 115.4 J K^{-1} mol^{-1}, respectively.

It was also possible, using transition-state theory, to determine the thermodynamic parameters for formation of the conformational transition state of the protein, when it is poised between open and closed conformations, from the relationships:

$$E_a = \Delta H^‡ + RT$$

$$k = \frac{k_b T e^{\Delta S^‡/R} e^{-(\Delta H^‡/RT)}}{h}$$

$$\Delta G^‡ = \Delta H^‡ - T\Delta S^‡$$

where k is either k_1, k_{-1}, or k_{-3}, E_a is the corresponding activation energy, k_b is Boltzmann's constant, h is Planck's constant, R is the gas constant, T is the absolute temperature, and $\Delta G^‡$, $\Delta H^‡$, and $\Delta S^‡$ are the standard Gibbs free energy, enthalpy, and entropy changes, respectively, associated with formation of the transition state. The calculated thermodynamic parameters for the formation of the transition state between open and closed conformations for the unliganded protein (k_{-1}) and for the formation of the closed to open transition state of the unliganded (k_1) and ligand-loaded (k_{-3}) protein are given in Table II.

Table II: Basic Thermodynamic Parameters for Conformational Transitions of DctP at 20 °C

rate constant	E_a (kJ mol^{-1})	$\Delta H^‡$ (kJ mol^{-1})	$\Delta S^‡$ (J K^{-1} mol^{-1})	$\Delta G^‡$ (kJ mol^{-1})
k_1	29.8	27.4	−132.1	66.1
k_{-1}	76.0	73.6	48.7	59.3
k_{-3}	27.6	25.2	−131.7	63.8

DISCUSSION

The most striking feature of the present investigation is the finding that the rate constants k_1 and k_{-3} have similar pH, ionic strength, and temperature dependencies. This strongly supports the model we have previously proposed for ligand binding to DctP (Walmsley et al., 1992). In this model, both k_1 and k_{-3} refer to a transition of the protein between closed and open conformations. The only difference between these rate constants is that k_{-3} is greater than k_1 under all the conditions studied, indicating that ligand binding accelerates the transition between conformations. Furthermore, the data would suggest that it is this conformational transition that is rate-limiting for ligand dissociation and that ligand binding is a rapid equilibrium process.

The equilibrium between the BP_1 and BP_2 conformations is clearly dependent upon the protonation state of two different groups in the two conformations. The pK_a values of 5.3 and 10.1 might represent glutamate and lysine residues, respectively. The pH dependence of k_1 and k_{-3} indicates that the deprotonated conformational forms interconvert more rapidly. Perturbation of the equilibrium between the two conformational forms, toward the open conformation at pHs above and below pH 8, is concomitant with a decrease in the ligand binding affinity of the BP_2 conformation. This suggests that these residues are directly involved in binding the ligand, possibly via an ionic interaction with the dicarboxylate ion. This would, however, contrast with studies of other anion binding proteins which have revealed that ligand binding largely involves hydrogen bonds rather than ionic interactions (Pflugrath & Quiocho, 1985; Leucke & Quiocho, 1990). Alternatively, the two groups could be involved in forming salt bridges, essential in stabilizing the BP_1 conformation at neutral pH. It is interesting to note that at neutral pH, where the closed conformation is favored, the carboxylate group of the putative glutamate residue would be deprotonated while the amino group of the putative lysine would be protonated. As such, these two groups would be capable of forming a salt bridge; the fact that we were unable to assign a given pK value to a particular conformational state of the protein is consistent with both residues being involved in such an interaction. Any

shift in pH from neutrality would have a tendency to destabilize this salt bridge and thus affect the stability of the closed conformation. The fact that the conformational equilibrium can also be perturbed by increasing ionic strength would support the supposition that these residues are involved in salt bridge formation. We have found that with increasing [NaCl] the rate constant for the closed to open transition increases hyperbolically to a maximal rate which is similar to that observed with increasing pH. This would suggest that the ionic strength effect is specific for these residues and is not merely causing a nonspecific destabilization of the protein. It is noteworthy that salt bridges have been identified in the tertiary structures of two other well-characterized anion binding proteins: the sulfate binding protein (between Glu15 and Arg174 and between Asp68 and Arg134; Plugrath & Quiocho, 1988) and the phosphate binding protein (Luecke & Quiocho, 1990). Ligand binding by the former protein has been shown to be pH-independent between pH 5.2 and 8.3, but is reduced at more extreme pH values, and, more importantly, sulfate binding is markedly dependent upon ionic strength (Pardee, 1966; Pardee et al., 1966). This would at first seem to be inconsistent with X-ray crystallographic studies, which have revealed that the ligand is held in the binding site entirely by hydrogen-bonding interactions (Pflugrath & Quiocho 1985, 1988), but is consistent with the destabilization of an ionic bond holding the protein in the binding conformation. More recently, the role of the two salt bridges in the sulfate binding protein has been investigated by site-directed mutagenesis (Jacobsen et al., 1992). The acidic residues Glu15 and Asp 68 were changed singly and together to residues with the corresponding amide side chains. These mutations produced only a moderate decrease in the affinity of the protein for sulfate but caused a dramatic increase in the dissociation rate, indicating that the salt bridges are important in stabilizing the closed conformation. Our data would suggest that such salt linkages may be a general feature of periplasmic anion binding proteins. One further observation worthy of comment with regard to DctP is the fact that in the presence of ligand the pK_a's for the two groups appear to be lowered by about 1 pH unit in each case. The significance of this finding is at present unclear, but it would suggest that the ligand either binds in the vicinity of these residues or causes a conformational change around them which lowers their pK_a's. This would have a tendency to destabilize the putative salt bridge at the pH optimum (pH 8.0), by increasing the protonation state of the high-pK_a residue, and consequently facilitate conformational interconversion. We also note that it is largely the interconversion rate of the protonated forms that is accelerated in the presence of ligand. Since the protonation state of both residues would be increased at pH 8.0, this would accelerate the rate and shift the equilibrium toward the open conformation. Although this might at first glance appear detrimental for high-affinity binding, it might be a necessary requirement for removal of the ligand from the binding protein after docking with the integral membrane protein component of the transport system (DctQ; M. J. Hamblin and D. J. Kelly, unpublished results).

The thermodynamic data obtained in this study indicate that the closed-unliganded conformation is stabilized by about 6.8 kJ mol^{-1} at 20 °C, pH 8. This can be readily overcome by the stabilization energy afforded by ligand binding to the open conformation ($\Delta G°$ = –40.9 kJ mol^{-1}). The closed-liganded conformation is stabilized by about a further 6.2 kJ mol^{-1} (Walmsley et al., 1992), so that the overall free energy change for ligand binding is about 41 kJ mol^{-1} (20 °C, pH 8). In the absence of ligand, the transition from the closed to the open conformation is characterized by enthalpy and entropy changes of –55.2 kJ mol^{-1} and –212 J K^{-1} mol^{-1}, respectively. The exothermic entropy change would indicate that bond formation is involved in adopting the open conformation. This is surprising, since this transition has been shown by X-ray crystallographic studies of other binding proteins to involve a large movement of the two protein domains away from one another (Quiocho, 1990). Breakage of the putative salt bridges holding the protein in the closed conformation would presumably lead to a positive change in the enthalpy. Furthermore, in adopting the open conformation the protein structure would become less constrained, which would tend to increase the entropy of the system. The observed decrease in enthalpy and the large decrease in entropy might be attributable to the solvation of residues which are buried within the protein structure in the closed conformation. As these residues become exposed to the solvent in adopting the open conformation, water molecules will become ordered around them. This will probably involve both the simple ordering of water molecules around hydrophobic residues and hydrogen bonding of water molecules with ionic residues. This would clearly decrease the entropy of the system, while hydrogen bond formation would give rise to a negative enthalpy change. For other binding proteins, which are thought to reside predominantly in the open conformation, the decrease in entropy due to water ordering around otherwise buried residues may be the most important thermodynamic effect in stabilizing the open conformation. In order for the protein to readopt the closed conformation, this ordered water must be displaced from the protein. This process is probably achieved by ligand binding. In the present study, we have shown that fumarate binding involves a large increase in entropy, possibly due to water displacement. A salt linkage is thought to have an energy of about 17 kJ mol^{-1} (Fersht, 1985), so that if a single salt bridge was involved in stabilizing the closed form of DctP, then the predicted enthalpy of solvation upon adopting the open conformation would be around 72 kJ mol^{-1}, i.e., the sum of the observed enthalpy change (–55 kJ mol^{-1}) and the enthalpy for breakage of this salt bridge (17 kJ mol^{-1}). This value is very similar to the positive enthalpy change observed for fumarate binding (74.7 kJ mol^{-1}). Formation of the closed conformation may be facilitated by displacement of this water. In this study, we have noted that the transition from the closed to open conformation is accelerated by bound ligand. In a previous study, we have shown that the rate of the open-liganded to closed-liganded transition is dependent upon the nature of the ligand (Walmsley et al., 1992). For L-malate, the ligand bound with highest affinity, the rate of this step is around 650 s^{-1}, 3–4 times faster than the analogous transition in the absence of ligand. This might be due to the ligand providing a more favorable hydrogen-bonding pathway for the conformational change. The activation energy for the closed to open transition is similar in the presence and absence of ligand, but with the ligand lowering the activation energy by about 2 kJ mol^{-1}. In contrast, the activation energy for the unliganded-open to unliganded-closed transition is nearly 3 times higher. Ligand binding may have the effect of reducing the energy barrier, possibly by reducing the entropy effect by water displacement and increasing the enthalpy effect by providing more favorable hydrogen bonds for the transition. It is interesting to note that in forming the transition state there is a decrease in the entropy in going from the closed to the open form but an increase in entropy in the reverse direction. This might suggest

that in going to the open conformation the protein adopts a more constrained structure in the transition state. On the other hand, it might be that this transition state represents a more solvated structure. Converse reasoning could be applied for the transition state formed on passage to the closed conformation. Although this interpretation constitutes a simple way of describing the chemical interactions that underlie the thermodynamics of ligand interaction with binding proteins, other explanations are possible. However, similar thermodynamic models have been suggested for other proteins which are involved in simple ligand binding phenomena, such as glucose binding to hexokinase (Janin & Wodak, 1983) and the glucose transporter (Walmsley, 1988). Moreover, the detailed X-ray crystallographic studies performed on other binding proteins point to the importance of water association and displacement in stabilizing and destabilizing the protein structure.

DctP may be of general importance in understanding the molecular events involved in the conformational changes associated with ligand binding, since this represents the only example, to date, of a binding protein where the kinetics of all the individual mechanistic steps can readily be measured.

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