Crowding by Trisaccharides and the 2:1 Cytochrome *c*−Cytochrome *c* Peroxidase Complex†

Artemiza S. Morar and Gary J. Pielak*

Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-3290

Received June 7, 2001; Revised Manuscript Received October 29, 2001

ABSTRACT: Proteins are designed to function under crowded conditions where the solute concentration can reach 400 g/L, but they are almost always studied in dilute solutions. To address this discrepancy, we have undertaken a series of studies to determine the effects of high solute concentrations on the thermodynamics of protein equilibria. Recently, we used isothermal titration calorimetry (ITC) to show that high concentrations of mono-, di-, and tetrasaccharides have a small stabilizing effect on the crystallographically defined cytochrome *c* binding site on yeast ferricytochrome *c* peroxidase [Morar, A. S., Wang, X., and Pielak, G. J. (2001) *Biochemistry 40*, 281−285]. Here, we use this technique to show that trisaccharides increase the apparent thermodynamic binding constants for both cytochrome *c* binding sites on the peroxidase. Mutagenesis studies confirm that the second site includes Asp 148 on the peroxidase. Binding of both cytochrome *c* molecules is exothermic. The data are interpreted by assuming either the presence or absence of intersite interactions.

The inside of the cell is a crowded place. Intracellular solutes can reach concentrations of 400 g/L and occupy up to 30% of the cell's volume (*1*). However, the solute concentration in most in vitro experiments is less than 1 mg/mL. This dichotomy between in vivo conditions and in vitro experiments may prevent a proper understanding of intracellular events such as protein aggregation and metabolic rates (*2*, *3*).

Cytochrome *c* peroxidase (CcP)[1] is a biological redox partner of cytochrome *c* (Cc) in yeast. Both proteins are found in the periplasmic space of the mitochondria, and the structure of the 1:1 Cc−CcP complex is known to high resolution (*4*). CcP catalyzes the oxidation of Cc by hydrogen peroxide:

$$2Cc^{2+} + H_2O_2 + 2H^+ \leftrightharpoons 2Cc^{3+} + 2H_2O$$

Peroxidase first undergoes a two-electron oxidation by hydrogen peroxide to form a radical-containing intermediate. This intermediate is reduced to the resting state by two Cc molecules in consecutive one-electron steps.

Our goal is to understand the effect of molecular crowding on the equilibrium thermodynamics of protein complex formation. Under certain conditions, CcP can bind two Cc molecules (*5*, *6*). Evidence for the second binding site comes from size exclusion chromatography, steady-state kinetics, photoinitiated electron transfer, and titration calorimetry (*7*, *8*). Brownian dynamics simulations suggest that the second site is located near residue Asp 148 of CcP (*7*, *9*). The estimated equilibrium constant for forming the 2:1 complex in dilute solution for both the horse Cc and yeast iso-1-Cc−CcP complexes is between 2×10^3 and 7×10^3 M^{-1} near 20 °C, pH 7, and low (≤50 mM) ionic strength (*5*, *8*). The 2:1 stoichiometry has not been observed at higher (>100 mM) ionic strengths (*10*). The physiological and catalytic relevance of the 2:1 complex is controversial (*11*−*13*), and several models have been proposed to reconcile thermodynamic and electron transfer data (*7*).

In all previous studies, binding at the second site was too weak to provide accurate thermodynamic parameters. We used ITC to show 1:1 binding for the yeast Cc−CcP reaction in dilute solution (*14*, *15*) and in solutions crowded with mono-, di-, and tetrasaccharides at sugar concentrations of up to 470 g/L (*16*). Here we use this technique to study complex formation in the presence of the trisaccharides melezitose and raffinose.

MATERIALS AND METHODS

Protein Purification. Recombinant yeast iso-1-Cc and recombinant yeast CcP were expressed and purified from *Escherichia coli* as described previously (*16*). CcP concentrations were determined at 408 nm by using a molar absorptivity of 102 mM^{-1} cm^{-1} (*17*). Cc concentrations were determined at 410 nm by using a molar absorptivity of 106.1 mM^{-1} cm^{-1} (*18*). The D148A mutant of CcP was constructed as described previously (*15*).

ITC. Experiments were performed on the ferri form of both proteins by using a Microcal MCS isothermal titration calorimeter. The trisaccharides melezitose and raffinose were purchased from Sigma and used without further purification. Solution preparations and the parameters used in titrations were identical to those used in our studies of mono-, di-, and tetrasaccharides (*16*). Thermodynamic parameters are quoted in terms of complex formation at a standard state of

† This work was supported by NIH Grant GM42501.

* To whom correspondence should be addressed. Phone: (919) 966-3671. Fax: (919) 966-3675. E-mail: gary_pielak@unc.edu.

[1] Abbreviations: Cc, cytochrome *c*; CcP, cytochrome *c* peroxidase; DMG, 3,3′-dimethylglutaric acid; ITC, isothermal titration calorimetry; tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

10.1021/bi0111810 CCC: $22.00 © 2002 American Chemical Society
Published on Web 12/15/2001

1 M reactants and products at 25 °C, 50 mM 3,3′-dimethylglutaric acid (DMG), and pH 6.0. The ionic strength of this buffer is ~80 mM (*19*). The calorimeter was calibrated by titrating 2′-CMP into ribonuclease A as recommended by Microcal.

Data were analyzed with Origin 5.0 (Microcal Software). Baseline and start and end points for integration were adjusted manually. Corrections for heats of dilution were made without correcting for the heat of buffer ionization. Both one- and two-site fitting algorithms were tested. For two-site binding, the "two independent sites" model and the "interacting sites" model were used. The binding free energy, ΔG_i, was calculated from the equation $\Delta G_i = -RT \ln K_i$, and the binding entropy, ΔS_i, was calculated from ΔG_i and ΔH_i by using the equation $\Delta G_i = \Delta H_i - T\Delta S_i$.

Steady-State Kinetics. The buffer, 20 mM KH_2PO_4, was adjusted to pH 6.0 with 2-amino-2-(hydroxymethyl)-1,3-propanediol (tris) as described by Fishel et al. (*20*). The melezitose concentration was 0.2 M. Stock solutions of CcP were made by dissolving CcP crystals in buffer. Cc solutions were reduced just prior to use by adding a small excess of $Na_2S_2O_4$. Excess $Na_2S_2O_4$ was then removed by gel filtration chromatography on a NAP-10 column (Pharmacia) equilibrated with KH_2PO_4/tris buffer. The percent reduction of Cc was at least 95% in all experiments. H_2O_2 solutions were made immediately before use by diluting a fresh 3% stock solution (Mallinckrodt). The final reaction volume was 1 mL, and the final concentrations were 2 nM CcP and 200 μM H_2O_2. Reactions were performed at Cc concentrations of 5, 10, 15, 20, 25, 30, 40, 50, and 60 μM and initiated by adding 6 μL of a CcP solution. The absorbance at 550 nm was monitored over 200 s at 25 °C with a Shimadzu BioSpec-1601 dual-beam spectrophotometer. Absorbance data were collected every 5 s. The initial velocity was obtained by using the equation $V_0 = \Delta A/(2\Delta E\Delta t)$, where ΔA is the change in absorbance, ΔE is the difference in molar absorptivity of reduced and oxidized Cc, and Δt is the time change. V_{max} and K_m were determined from linear regression analysis of Lineweaver−Burk plots. Monophasic kinetics were observed under all conditions.

RESULTS

Data Fitting. ITC measures heat changes caused by the interaction of molecules in solution (*21*). The heats generated by successive injections are plotted against the mole ratio of Cc to CcP to obtain the binding isotherm, which is then fitted to a model (Figure 1). The fitting program returns values for the binding constant, K_i, the enthalpy of binding, ΔH_i, and the stoichiometry (moles of Cc per mole of CcP), N_i. To obtain these parameters by using the Microcal software, a number of iterations are performed until the fractional change in χ^2 is smaller than the tolerance value and ceases to decrease. A χ^2 value of 3×10^3 indicates a good fit. In all instances, the final fitting uncertainty is smaller than the uncertainty from repeating experiments, suggesting that data are consistent with the model.

Stoichiometry. Data for dilute solutions and sugars other than trisaccharides (*16*) are well fit by using a one-site model ($\chi^2 < 3 \times 10^3$). Data for trisaccharides are not well fit by using the one-site model ($\chi^2 = 2-6 \times 10^4$). For these sugars, the quality of the fit was improved by using two-site models.

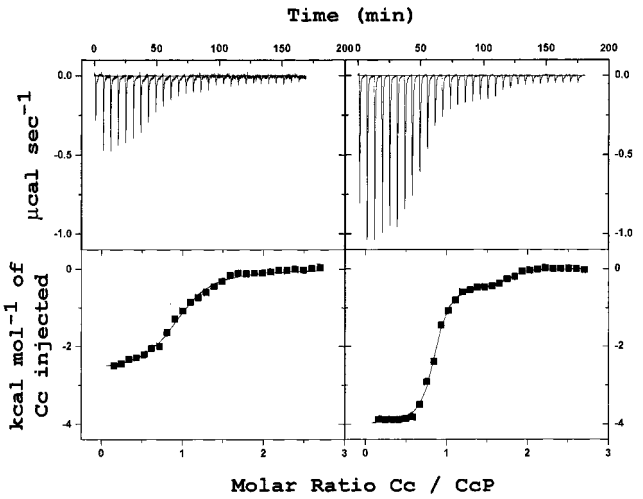

FIGURE 1: ITC data showing the interaction between Cc and CcP at 25 °C and pH 6.0, with 50 mM DMG. The top panels show data after baseline correction. The bottom panels show integrated heats and the best fit curve by using 1:1 (dilute) or 2:1 (melezitose) binding models. Left panels show data for a dilute solution (1.06 mM Cc was titrated into 53.5 μM CcP, with an injection volume of 6 μL), and the right panels show data for 0.7 M melezitose (1.16 mM Cc was titrated into 94.8 μM CcP, with an injection volume of 9 μL).

Stoichiometries for both sites range between 0.83 ± 0.02 and 1.19 ± 0.26 (Table 1). The nonunitary stoichiometries probably arise from systematic errors in Cc and CcP concentrations (*21*).

We tried both the "two sets of sites" model and the "sequential binding site" model. For the two sets of sites model, all parameters are floated. For the sequential binding sites model, N_1 and N_2 are fixed at unity. The χ^2 value from the two sets of sites model (8×10^2 to 3×10^3) is less than that from the sequential binding sites model ($< 7 \times 10^3$). Nevertheless, when we started with the same initial parameters, we arrived at the same final parameters with both models. This identity supports an idea that the trisaccharides stabilize the second binding site.

Reverse Titration. To saturate the low-affinity binding site, a reverse titration was carried out in 0.7 M melezitose. For this experiment, a 0.22 mM Cc solution was placed in the cell and titrated with 28 μM CcP. Once the CcP to Cc ratio reaches 0.5 (corresponding to a 2:1 Cc:CcP complex molar ratio), further addition of CcP leads to the 1:1 complex. That is, Cc is transferred from the low-affinity site to the high-affinity site. As expected for a 2:1 Cc−CcP complex, a minimum in the titration curve was reached at a CcP:Cc ratio of 0.5 (Figure 2). This result also supports a 2:1 binding model.

Thermodynamics of Cc Binding. The binding enthalpy for both Cc molecules is exothermic, but complex formation is mostly driven by a large and favorable entropy change (Table 1). Furthermore, the binding of the first Cc molecule is more exothermic ($\Delta H_1^{\circ\prime\prime} = -2.8$ to -4.3 kcal/mol) than the binding of the second ($\Delta H_2^{\circ\prime\prime} = -0.3$ to -0.6 kcal/mol). The apparent stabilizing effect of trisaccharides on the binding of the first Cc molecule is larger (≥3.5 kcal/mol) than the effects of sugars for which only the 1:1 complex is detected [≤0.5 kcal/mol (*16*)]. Furthermore, increasing the sugar concentration has only a small effect on $\Delta G_1^{\circ\prime\prime}$ and $\Delta G_2^{\circ\prime\prime}$, and there is no clear dependence on sugar concentra-

Table 1: Effects of Trisaccharides on the Thermodynamic Parameters for Formation of the Cc−CcP Complex[a]

sugar	[sugar] (M)	N_1	$\Delta H_1^{\circ\prime\prime}$	$-T\Delta S_1^{\circ\prime\prime}$	$\Delta G_1^{\circ\prime\prime}$	N_2	$\Delta H_2^{\circ\prime\prime}$	$-T\Delta S_2^{\circ\prime\prime}$	$\Delta G_2^{\circ\prime\prime}$
none	0	1.04 ± 0.15	−2.3 ± 0.4	−5.3 ± 0.5	−7.7 ± 0.1	–	–	–	–
melezitose	0.2	0.93 ± 0.002	−3.7 ± 0.1	−6.3 ± 0.3	−10.0 ± 0.2	0.84 ± 0.02	−0.6 ± 0.1	−7.4 ± 0.2	−8.0 ± 0.2
	0.45	0.90 ± 0.18	−3.1 ± 0.3	−6.9 ± 0.2	−10.1 ± 0.4	0.98 ± 0.14	−0.3 ± 0.2	−8.3 ± 0.1	−8.6 ± 0.2
	0.7	0.83 ± 0.02	−4.3 ± 0.2	−7.0 ± 0.1	−11.3 ± 0.2	1.19 ± 0.26	−0.5 ± 0.03	−8.1 ± 0.2	−8.6 ± 0.2
raffinose	0.3	0.89 ± 0.07	−2.8 ± 0.1	−8.5 ± 0.2	−11.3 ± 0.3	0.92 ± 0.12	−0.3 ± 0.1	−8.4 ± 0.5	−8.7 ± 0.4

[a] Experiments were performed at pH 6.0 and 25 °C, in 50 mM DMG. $\Delta H^{\circ\prime\prime}$, $-T\Delta S^{\circ\prime\prime}$, and $\Delta G^{\circ\prime\prime}$ are in kilocalories per mole. N is the stoichiometry (Cc:CcP). Trisaccharide data are the average of two titrations, and their uncertainties are given as average deviations. Data for the wild type in the absence of sugar are from Morar et al. (*16*).

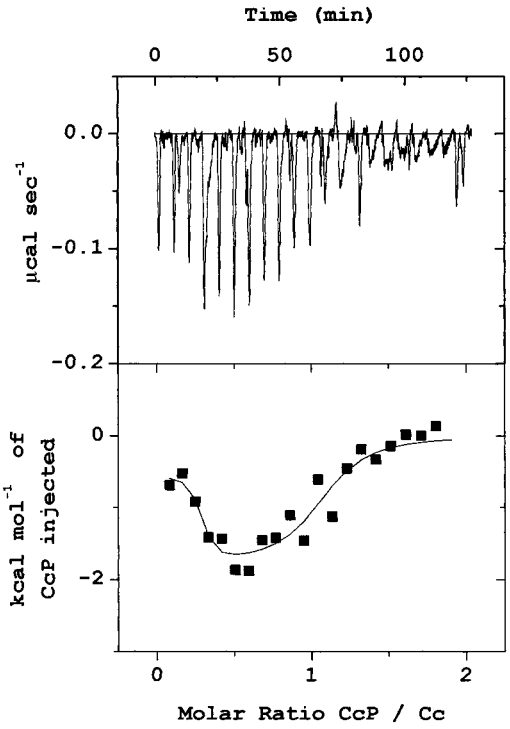

FIGURE 2: Reverse titration ITC data for the interaction between CcP and Cc at 25 °C and pH 6.0, with 50 mM DMG and 0.7 M melezitose. The top panel shows data after baseline correction. The bottom panel shows integrated heats (■) and the curve (—) of the best fit to a 1:2 binding model (28 μM cytochrome *c* was titrated with 0.22 mM peroxidase, with an injection volume of 14 μL).

Table 2: Effects of Trisaccharides on the Thermodynamic Parameters for Formation of the Cc−D148A CcP Complex[a]

sugar	[sugar] (M)	N	$\Delta H^{\circ\prime\prime}$	$-T\Delta S^{\circ\prime\prime}$	$\Delta G^{\circ\prime\prime}$
none	0	1.02 ± 0.02	−2.2 ± 0.4	−5.6 ± 0.2	−7.7 ± 0.2
melezitose	0.2	0.87 ± 0.04	−3.3 ± 0.4	−4.6 ± 0.6	−7.8 ± 0.3

[a] Data for the D148A variant are the average of two titrations for dilute conditions and four titrations for crowded conditions, and their uncertainties are given as average deviations and standard deviations, respectively. Other information is the same as that given in the footnote of Table 1.

tion, although sugars affect both $\Delta H^{\circ\prime\prime}$ and $\Delta S^{\circ\prime\prime}$. This observation of a nearly constant stability with increasing trisaccharide concentrations is consistent with the idea that sugar-induced crowding has only a small effect on complex stability, and agrees with our observations where only 1:1 binding was detected (*16*).

D148A Variant of CcP. The thermodynamic parameters for the interaction of this variant with wild-type Cc (Table 2) are similar to those for the complex between the wild-type proteins in dilute solution (Table 1). Furthermore, the parameters are not affected by trisaccharides. These results suggest that the second site is near Asp 148.

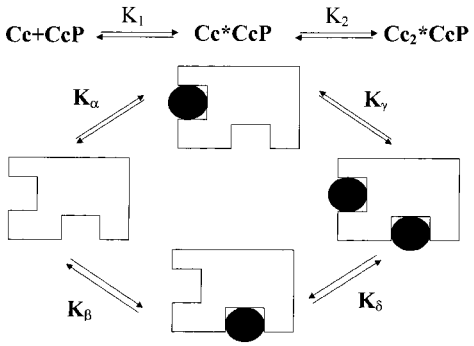

FIGURE 3: Thermodynamic and site binding models.

DISCUSSION

Why are two Cc binding sites detected with ITC in trisaccharide solutions, but only one site is detected with ITC in dilute solution and in solutions of mono-, di-, and tetrasaccharides? Given that a second site is observed with other techniques in dilute solution, it is likely that the second site is present in all sugar solutions, but trisaccharides increase the magnitude of the binding enthalpy and/or the binding free energy, allowing detection of the second site by ITC.

Binding Models. ITC measures the stoichiometric constants shown at the top of Figure 3, but these constants provide no information about binding at individual sites or intersite interactions. To obtain information about individual sites, we employed the site binding model (*22*) shown at the bottom of Figure 3. The two sites are different, so there are two ways to form the 2:1 complex. Four affinity constants (K_α, K_β, K_γ, and K_δ) characterize this model. Stoichiometric and site binding constants are related:

$$K_1 = K_\alpha + K_\beta$$

$$K_1K_2 = K_\alpha K_\gamma = K_\beta K_\delta$$

A unique solution for all four site constants cannot be obtained, but partial solutions come from applying either of two simplifying assumptions (*22*).

Assumption 1: The Affinity of the First Site Does Not Change with the Occupancy of the Second Site. This model involves binding at two independent domains, where "independent" means that the binding constant for one site is the same whether the other site is occupied. In this instance, $K_\alpha = K_\delta$ and $K_\beta = K_\gamma$, and unique values for K_α and K_β can be

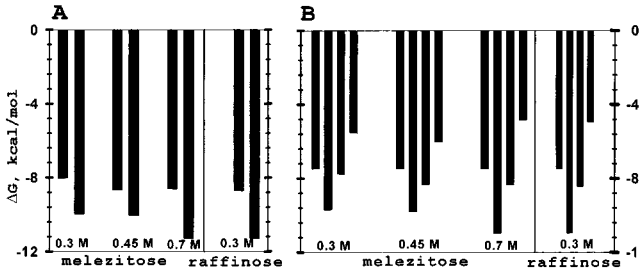

FIGURE 4: Site binding constants. (A) ΔG does not change with occupancy ($K_\alpha = K_\delta$, $K_\beta = K_\gamma$). For each entry, the bar on the left represents ΔG_α and the bar on the right ΔG_β. (B) ΔG changes with occupancy ($K_\alpha \neq K_\delta$, $K_\beta \neq K_\gamma$). For each entry, the bars, from left to right, represent ΔG_α, ΔG_β, ΔG_γ, and ΔG_δ.

obtained from the above equations. The results of this analysis are shown in Figure 4A. Binding is stronger at the site described by ΔG_β than it is at the site described by ΔG_α. Also, ΔG_α (at least -8 kcal/mol) is approximately equal to the $\Delta G^{\circ\prime\prime}$ value obtained in dilute solution (Table 1) and in sugar solutions where only a 1:1 complex is observed (*16*). These observations can be interpreted in two ways. If α represents the crystallographically defined site, trisaccharides have only a small effect on binding at the crystallographically defined site, but a large effect on binding at the second site. If β represents the crystallographically defined site, then trisaccharides strengthen binding at both sites.

Assumption 2: The Affinity of the First Site Changes with the Occupancy of the Second Site. This assumption seems reasonable because CcP is too small to bind two Cc molecules without some intersite interaction, especially because electrostatics play a large role in complex stability (*4*). To utilize this model, we assume that the site described by K_α is unaffected by sugars. That is, we assume that K_α equals the binding constant obtained in dilute solution (K_{dil}) so that values for the other site binding constants can be obtained as follows:

$$K_\beta = K_1 - K_{dil}$$

$$K_\gamma = (K_1 K_2)/K_{dil}$$

$$K_\delta = (K_{dil} K_\gamma)/K_\beta$$

The results are shown in Figure 4B. The K_γ/K_β and K_δ/K_α ratios measure the interaction between Cc molecules bound at the two sites. These ratios are less than unity, suggesting that the molecules bound at the two sites repel each other, as is expected for the binding of two cationic proteins. The interaction free energy [$-RT \ln(K_\gamma/K_\beta)$ or $-RT \ln(K_\delta/K_\alpha)$] is $\sim$1$-$3 kcal/mol, slightly larger than the value reported by Leesch et al. for the horse Cc$-$CcP complex (*8*).

Locating the Second Site. Changing Asp 148 on CcP to Ala restores monovalent binding, and has only a small effect on the thermodynamic parameters for monovalent binding (Table 2). Data from our previous ITC studies show that Asp 148 is not part of the high-affinity site in dilute solution (*15*). However, in a study of a different Cc, Leesch et al. (*8*) showed that Lys 149 is at or near the low-affinity site.

Origin of the Second Site. The observation that trisaccharides, but not mono-, di-, or tetrasaccharides, reveal the second site suggests the presence of specific interactions between trisaccharides and Cc or CcP. To test this suggestion, we looked for specific interactions between melezitose and Cc and CcP. ^{1}H NMR data from experiments based on the work of Yi et al. (*23*) suggest that melezitose does not affect the heme environment of the CN complex of CcP (A. S. Morar, G. B. Young, and G. J. Pielak, unpublished observations). Pulsed-field gradient NMR data from experiments based on the work of Wilkins et al. (*24*), ^{1}H$-^{15}$N chemical shift data, and ^{1}H$-^{15}$N redox shift data from experiments based on the work of Boyd et al. (*25*) all show that melezitose does not affect Cc structure (A. S. Morar, G. B. Young, and G. J. Pielak, unpublished observations). These results are consistent with the idea that sugars bind minimally to proteins (*26*).

Table 3: Effects of Melezitose on the Steady-State Kinetic Parameters for H_2O_2-Induced Cc Oxidation by CcP at pH 6.0 and 25 °C

	yeast Cc[a]	recombinant Cc[b]	
	dilute	dilute	melezitose
k_{cat} (s^{-1})	461 ± 65	386 ± 35	33 ± 11
K_m (μM)	180 ± 33	344 ± 32	36 ± 13

[a] From Miller et al. (*29*); KH_2PO_4/NaCl, 20 mM ionic strength. [b] From this study; 20 mM KH_2PO_4/tris.

Having failed to detect specific binding, we looked for more subtle effects. All the sugars studied here and in our previous work (*16*) contain a nonreducing terminal glucose or fructose, but only the trisaccharides melezitose and raffinose have the right length and the correct stereochemistry to juxtapose the two glycopyranosyl rings. Consequently, trisaccharides may behave as branched, polyfunctional saccharides (*27*). This behavior could alter the pattern of H bonds at the protein surface, inducing a subtle chain in one or both of the proteins.

Implications for CcP Activity. Crowding decreases k_{cat} and K_m (Table 3). Both parameters are 10-fold smaller in melezitose than they are in dilute solution. One of the binding site models (*28*) predicts that Cc bound at the second site is more reactive than the one bound at the high-affinity site. Our kinetic data do not provide information about the reactivity at the two sites, but we can conclude that crowding decreases the turnover rate.

CONCLUSIONS

High concentrations of trisaccharides allow ITC to detect the second Cc binding site on CcP. Analysis of the binding data shows that the second site includes Asp 148 on CcP. We cannot account for the ability of trisaccharides to stabilize the second site, but we have shown that specific trisaccharide binding does not seem to be involved. However, additional studies are needed to address the temperature, pH, and ionic strength dependencies of Cc$-$CcP complex formation in trisaccharide solutions. Such studies would provide a more comprehensive understanding of the subtle trisaccharide effect and the nature of the second site.

Overall, the data presented here show that sugar concentrations which mimic the solute concentrations found in the cell can shape protein$-$protein reactions. Our results indicate that the effects of crowding cannot be ignored.

ACKNOWLEDGMENT

We thank Thomas Poulos for supplying the CcP expression system, Grant Mauk for the Cc expression system, Xuming Wang for purifying some of the CcP, the Pielak lab for helpful discussions, and an anonymous reviewer for providing insight into the binding models.

REFERENCES

1. Zimmerman, S. B., and Trach, S. O. (1991) *J. Mol. Biol. 222*, 599−620.
2. Endy, D., and Brent, R. (2001) *Nature 409*, 391−395.
3. Minton, A. P. (2001) *J. Biol. Chem. 276*, 10577−10580.
4. Pelletier, H., and Kraut, J. (1992) *Science 258*, 1748−1755.
5. Mauk, M. R., Ferrer, J. C., and Mauk, A. G. (1994) *Biochemistry 33*, 12609−12614.
6. Kang, C. H., Ferguson-Miller, S., and Margoliash, E. (1977) *J. Biol. Chem. 252*, 919−926.
7. Nocek, J. M., Zhou, J. S., De Forest, S., Priyadarshy, S., Beratan, D. N., Onuchic, J. N., and Hoffman, B. M. (1996) *Chem. Rev. 96*, 2459−2489.
8. Leesch, V. W., Bujons, J., Mauk, A. G., and Hoffman, B. M. (2000) *Biochemistry 39*, 10132−10139.
9. Northrup, S. H., Luton, J. A., Boles, J. O., and Reynolds, J. C. (1988) *J. Comput.-Aided Mol. Des. 1*, 291−311.
10. Mei, H., Wang, K., McKee, S., Wang, X., Waldner, J. L., Pielak, G. J., Durham, B., and Millett, F. (1996) *Biochemistry 35*, 15800−15806.
11. Mei, H., Wang, K., Peffer, N., Weatherly, G., Cohen, D. S., Miller, M., Pielak, G., Durham, B., and Millett, F. (1999) *Biochemistry 38*, 6846−6854.
12. Miller, M. A., Geren, L., Han, G. W., Saunders, A., Beasley, J., Pielak, G. J., Durham, B., Millett, F., and Kraut, J. (1996) *Biochemistry 35*, 667−673.
13. Pappa, H. S., Tajbaksh, S., Saunders, A. J., Pielak, G. J., and Poulos, T. L. (1996) *Biochemistry 35*, 4837−4845.
14. Wang, X., and Pielak, G. J. (1999) *Biochemistry 38*, 16876−16881.
15. Pielak, G. J., and Wang, X. (2001) *Biochemistry 40*, 422−428.
16. Morar, A. S., Wang, X., and Pielak, G. J. (2001) *Biochemistry 40*, 281−285.
17. Dasgupta, S., Rousseau, D. L., Anni, H., and Yonetani, T. (1989) *J. Biol. Chem. 264*, 654−662.
18. Margoliash, E., Frohwirt, N., and Weiner, E. (1959) *Biochem. J. 71*, 550−572.
19. Eberson, L., and Wadsö, I. (1963) *Acta Chem. Scand. 17*, 1552−1562.
20. Fishel, L. A., Villafranca, J. E., Mauro, M., and Kraut, J. (1987) *Biochemistry 26*, 351−360.
21. Blanamer, M. (1998) in *Biocalorimetry: Applications of Calorimetry in the Biological Sciences* (Ladbury, J. E., and Chowdhry, B. Z., Eds.) pp 5−25, Wiley, Chichester, U.K.
22. Klotz, I. M. (1997) *Ligand-receptor energetics*, Wiley, New York.
23. Yi, Q., Erman, J. E., and Satterlee, J. D. (1994) *J. Am. Chem. Soc. 116*, 1981−1987.
24. Wilkins, D. K., Grimshaw, S. B., Receveur, V., Dobson, C. M., Jones, J. A., and Smith, L. J. (1999) *Biochemistry 38*, 16424−16431.
25. Boyd, J., Dobson, C. M., Morar, A. S., Williams, R. J. P., and Pielak, G. J. (1999) *J. Am. Chem. Soc. 121*, 9247−9248.
26. Timasheff, S. N. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 67−97.
27. Mulloy, B., Frenkiel, T. A., and Davies, D. B. (1988) *Carbohydr. Res. 184*, 39−46.
28. Kim, K. L., Kang, D. S., Vitello, L. B., and Erman, J. E. (1990) *Biochemistry 29*, 9150−9159.
29. Miller, M. A., Vitello, L., and Erman, J. E. (1995) *Biochemistry 34*, 12048−12058.

BI0111810