Sugar-Binding and Crystallographic Studies of an Arabinose-Binding Protein Mutant ($Met^{108}Leu$) That Exhibits Enhanced Affinity and Altered Specificity[†,‡]

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ABSTRACT: In addition to hydrogen bonds, van der Waals forces contribute to the affinity of protein–carbohydrate interactions. Nonpolar van der Waals contacts in the complexes of the L-arabinose-binding protein (ABP) with monosaccharides have been studied by means of site-directed mutagenesis, equilibrium and rapid kinetic binding techniques, and X-ray crystallography. ABP, a periplasmic transport receptor of *Escherichia coli*, binds L-arabinose, D-galactose, and D-fucose with preferential affinity in the order of Ara > Gal ≫ Fuc. Well-refined, high-resolution structures of ABP complexed with the three sugars reveal that the structural differences in the ABP–sugar complexes are localized around C5 of the sugars, where the equatorial H of Ara has been substituted for CH_3 (Fuc) or CH_2OH (Gal). The side chain of Met^{108} undergoes a sterically dictated, ligand-specific, conformational change to optimize nonpolar interactions between its methyl group and the sugar. We found that the $Met^{108}Leu$ ABP binds Gal tighter than wild-type ABP binds Ara and exhibits a preference for ligand in the order of Gal ≫ Fuc > Ara. The differences in affinity can be attributed to differences in the dissociation rates of the ABP–sugar complexes. We have refined at better than 1.7-Å resolution the crystal structures of the $Met^{108}Leu$ ABP complexed with each of the sugars and offer a molecular explanation for the altered binding properties.

The monomeric L-arabinose-binding protein (ABP)[1] is an excellent system for structure–function studies, especially in understanding molecular recognition of carbohydrate. ABP is a very stable protein and can be easily purified in gram quantities. The gene for ABP has been cloned and sequenced (Scripture et al., 1987), and a suitable expression system in *Escherichia coli* has been developed for the production of wild-type and mutant proteins (Horazdovsky & Hogg, 1987; Vermersch et al., 1990). Sugar binding can be easily analyzed by using equilibrium and rapid kinetic techniques (Miller et al., 1983; Vermersch et al., 1990). Crystals of ABP can be easily obtained and frequently diffract to better than 1.7-Å resolution. Finally, the structures of ABP complexed with various monosaccharide ligands have been refined at very high resolutions (Quiocho & Vyas, 1984; Quiocho et al., 1989). ABP consists of two globular domains bisected by a cleft wherein the sugar ligand is bound and completely sequestered.

ABP mediates the high-affinity uptake of L-arabinose in *E. coli* (Hogg & Englesberg, 1969; Schleif, 1969). With reduced affinity, ABP also binds D-galactose (approximately 2-fold reduction) and D-fucose (approximately 40-fold reduction) (Miller et al., 1983). Differences in the affinities are attributed to differences in the dissociation rate constants (Miller et al., 1983; Vermersch et al., 1990). Well-refined, high-resolution structures of ABP, complexed with the three sugars, have provided insight into the molecular basis of this specificity (Quiocho et al., 1989). In all three complexes, the sugar ring is positioned identically in the binding site of ABP. Each hydroxyl that is common to the three sugars (OH1α or -β, OH2, OH3, and OH4) participates in identical hydrogen-bonding interactions in the three structures. The sugars differ at C5, where the equatorial H of Ara has been substituted for CH_3 (in Fuc) or CH_2OH (in Gal).

There are two residues and one ordered water molecule that interact with the sugar and rearrange to confer specificity, Asp^{89}, Met^{108}, and Wat^{310}. Wat^{310} and the carboxylate side chain of Asp^{89} have moved in the ABP–Gal complex, relative to their positions in the ABP–Ara and ABP–Fuc complexes, to form a hydrogen bond with OH6. The side chain of Met^{108} undergoes a ligand-specific conformational change to optimize nonpolar interactions between its methyl group and the sugar (Figure 1). When Ara is bound, the Met^{108} methyl "swings" toward the sugar to optimize a nonpolar van der Waals contact at C5. Relative to Ara binding, the bulky C6 groups of Fuc and Gal cause a rotation of the methionine side chain. This sterically necessary, altered conformation creates favorable nonpolar contacts between the ϵ-methyl group of Met^{108} and C6 of Gal and Fuc.

We describe experiments designed to explore the effect of eliminating this conformational difference between the three ABP–sugar complexes at position 108. Using site-directed mutagenesis, we replaced Met^{108} with Leu. This mutation serves to remove the steric requirement for the side chain at position 108 to swing away from the sugar upon Fuc or Gal binding as well as the potential for it to swing toward the sugar

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[‡] The protein coordinates in this paper have been submitted to the Brookhaven Protein Data Bank.

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[1] Abbreviations: ABP, L-arabinose-binding protein; Ara, L-arabinose; Fuc, D-fucose; Gal, D-galactose; rms, root mean square.

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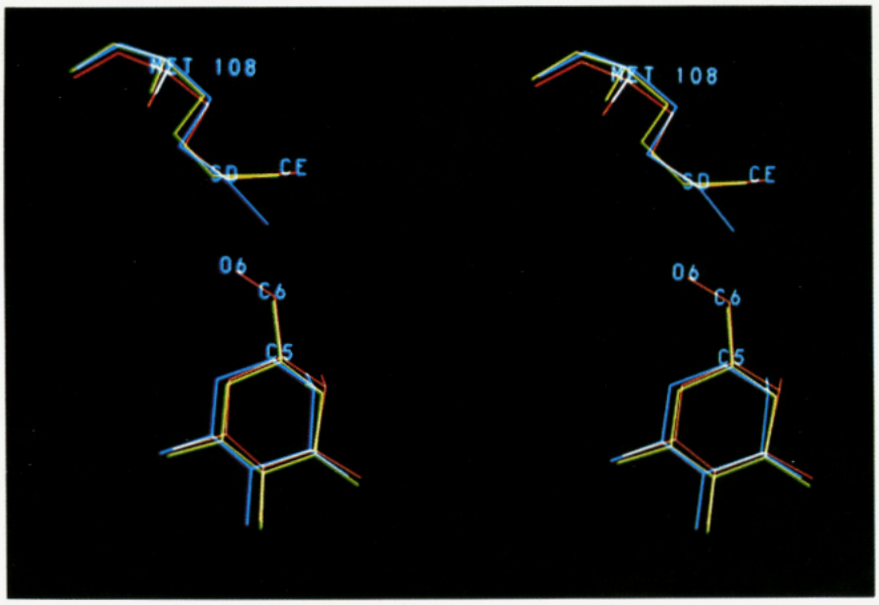

FIGURE 1: Conformation of Met[108] in wild-type ABP complexed with Ara, Gal, and Fuc. The structures of complexes with Ara (blue), Gal (red), and Fuc (yellow) are superimposed. For clarity, only one anomeric form of the sugars is shown for each complex. Atoms O6, C6, and C5 are labeled for the sugars, as appropriate.

upon Ara binding. The effects of the mutation on kinetic and equilibrium binding and the X-ray crystallographic structure have been determined for each of the three sugars.

Experimental Procedures

Materials. L-Arabinose, D-galactose, and D-fucose were obtained from Pfanstiehl Laboratories. D-[1-^{14}C]Galactose was obtained from Amersham. Oligonucleotides used for DNA mutagenesis and sequencing were synthesized at Howard Hughes Medical Institute (Houston) on an Applied Biosystems 380B DNA synthesizer or obtained commercially from OCS Laboratories, Inc.

Oligonucleotide-Directed Mutagenesis of the AraF Gene. The substitution of Leu for Met at position 108 was introduced into ABP by the method of Kunkel (1985). Uracil-containing single-stranded MPVA-1 DNA (Vermersch et al., 1990) was used as a template for primer extension with the mutagenic oligonucleotide. Codon ATG (Met) was replaced with TTG (Leu) at position 108 by using 5′-TAGTCGCCGCCAACATCACCAGC-3′. Selection against the nonmutant strand was achieved by transformation of JM101 (New England BioLabs, Inc.) with the product of the mutagenesis reaction. Mutants were identified by sequencing single-stranded DNA from several of the transformants. DNA was sequenced by the dideoxy method using a Sequenase kit from U.S. Biochemical Corp. Phage were plaque-purified and reconfirmed by DNA sequence analysis to contain the desired mutation.

Expression and Purification of Wild-Type and Mutant ABPs. The *Eco*RI/*Hin*dIII fragment containing the mutated *araF* gene was cloned into the *Eco*RI and *Hin*dIII sites of pIBI24 (International Biotechnologies, Inc.). *E. coli* strain CW2621 (*ara*E201, *ara*ΔFGH::Kan, *srl*::TN10, *rec*A59, *pro*), kindly provided by Dr. R. W. Hogg (Case Western University Medical School), was used for expression of the mutant ABP. Mutant and wild-type ABPs were isolated and rid of bound sugar as previously described (Vermersch et al., 1990).

Equilibrium Binding Assays. The fluorescence decrease at 370 nm was titrated by the addition of microliter aliquots of a concentrated sugar solution to 2 mL of 0.1–0.6 μM ABP. Fluorescence titrations were performed in a solution containing 10 mM Tris-HCl and 0.1 mM dithiothreitol, pH 7.4 at 20 °C (Miller et al., 1983; Vermersch et al., 1990).

Equilibrium dialysis binding assays were performed essentially as in Newcomer et al. (1979). ABP, 0.5 mL (5 μM), was dialyzed against 50 mL of buffer containing a fixed concentration of D-[1-^{14}C]galactose (0.33 μM) and a range of arabinose concentrations (0.001–1 mM). Protein concentration, determined with the Bio-Rad colorimetric assay and ABP of known concentration as a standard, was corrected for dilution.

Kinetic Analysis. Kinetic experiments were performed in a Gibson-Durrum stopped-flow rapid-mixing apparatus equipped for fluorescence measurements (Miller et al., 1983; Vermersch et al., 1990). Experiments were performed in a solution containing 10 mM Tris-HCl and 0.1 mM dithiothreitol, pH 7.4 at 20 °C. In the determination of association rate constants, pseudo-first-order conditions were maintained. Dissociation rate constants for the different ABP–sugar complexes were obtained by displacement with a second sugar having the appropriate spectral properties (Vermersch et al., 1990).

Crystallographic Analysis. Complexes of the Met[108]Leu ABP with D-galactose, D-fucose, or L-arabinose were crystallized and harvested as previously described (Vermersch et al., 1990). Cyrstals of mutant ABP–sugar complexes were isomorphous with crystals of wild-type ABP–Ara (Quiocho & Vyas, 1984; Quiocho et al., 1989). High-resolution X-ray data were collected at 20 °C with a SDMS two area detector system mounted on a Rigaku RU200 rotating anode X-ray generator (graphite-monochromated Cu Kα radiation at 4.4 kW). During data collection, alignment angles were automatically refined every 2.5° in ω to account for any crystal slippage. After data were processed with the Nielson software package, electron density maps were calculated by using the coefficients $F_{mutant} - F_{wild\text{-}type}$ and $2F_{mutant} - F_{wild\text{-}type}$ and the phases of the refined structure of the specific wild-type protein–sugar complex with the contribution of the Met[108] side chain omitted. These maps revealed well-resolved density in the area of the mutation, enabling easy fitting of the leucine side chain. Restrained least-squares refinement of the atomic coordinates against the X-ray data was carried out with the PROFFT version (Finzel, 1987) of PROLSQ (Hendrickson, 1985).

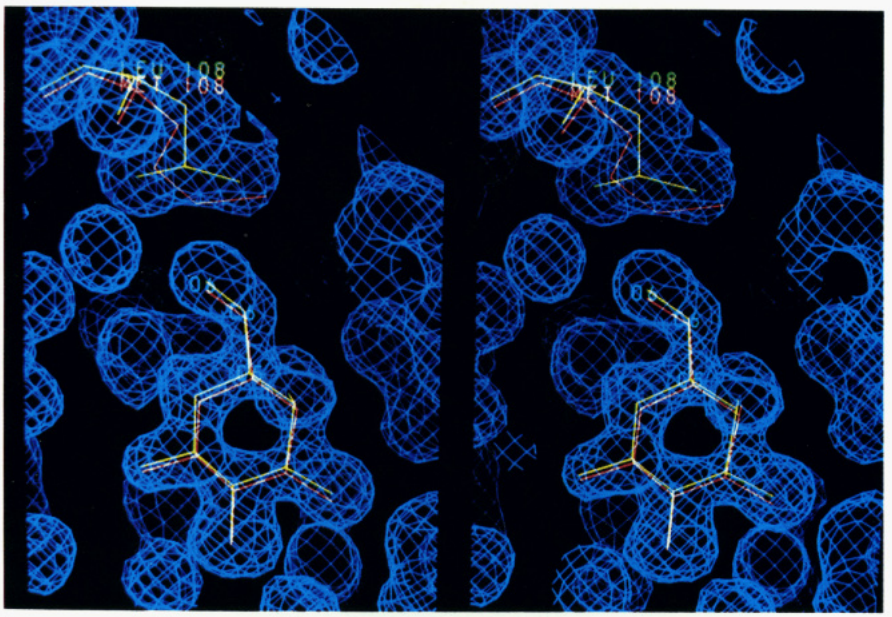

FIGURE 2: Electron density map at 1.49 Å of the Met[108]Leu ABP–Gal complex in the region of the Met to Leu mutation. The map, calculated with $2|F_o| - |F_c|$ coefficients and calculated phases, was generated with a 0.5-Å grid and contoured at 1.0 standard deviation of the map density. Structures of the Met[108]Leu (yellow) and wild-type (red) ABP–Gal complexes are superimposed. Note the density at the site of the Met to Leu mutation. Only one anomeric form of galactose is shown for each complex. Galactose atoms O6, C6, and C5 are labeled.

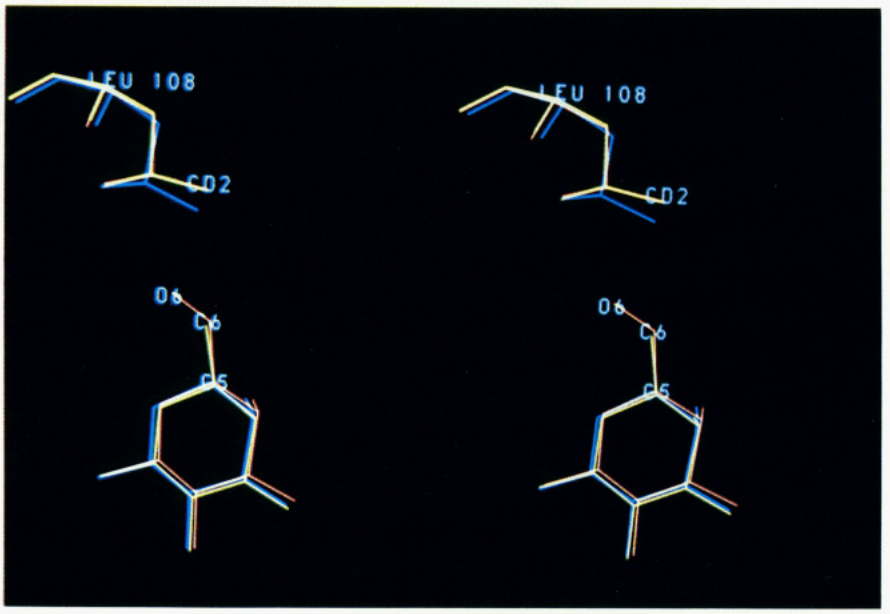

FIGURE 3: Conformation of Leu[108] in the Met[108]Leu ABP complexed with Ara, Gal, and Fuc. The structures of complexes with Ara (blue), Gal (red), and Fuc (yellow) are superimposed. Only one anomeric form of the sugars is shown for each complex. Atoms O6, C6, and C5 are labeled for the sugars, as appropriate.

All model building was done on an Evans and Sutherland PS390, using the density fitting and molecular modeling program CHAIN, developed in our laboratory (Sack, 1988).

Results and Discussion

Equilibrium Binding Studies. A blue spectral shift and fluorescence quenching are observed when ABP binds L-arabinose or D-fucose (Clark et al., 1982; Miller et al., 1983). When ABP binds D-galactose, the same spectral shift is observed; however, there is little apparent quenching (Clark et al., 1982). The Met[108]Leu ABP shows similar spectral properties; however, some quenching is also observed when D-galactose is bound. The fluorescence decrease at 370 nm was used to monitor L-arabinose and D-fucose binding in equilibrium titration experiments (Miller et al., 1983; Vermersch et al., 1990). K_d values were obtained from the slopes of Scatchard plots. Arabinose is bound by the Met[108]Leu ABP with about 5–10 times lower affinity than wild-type ABP. The affinity for fucose, however, was determined to be 10-fold greater for the mutant than for wild type (Table I).

The binding of D-galactose by the Met[108]Leu ABP was significantly tighter than that observed for the wild-type protein. A more accurate K_d was obtained by equilibrium dialysis competition experiments in which [^{14}C]galactose was displaced with arabinose. The ratio of K_d values (Gal:Ara) was determined to be 0.023. The affinity for the Met[108]Leu ABP for galactose is about 20-fold greater than that for wild-type ABP (Table I). Thus the preferential affinity of ABP for sugar (Ara > Gal ≫ Fuc) has been altered by the mutation of Met to Leu at position 108 (Gal ≫ Fuc > Ara).

Table I: Association Rate (k'), Dissociation Rate (k), and Equilibrium Binding (K_d) Constants for the Reaction of ABP and Met[108]Leu ABP with L-Arabinose, D-Galactose, and D-Fucose

protein and sugar	k' (μM^{-1} s^{-1})	k (s^{-1})	k/k' ($\times 10^6$ M)	K_d ($\times 10^6$ M)	ΔG (kcal mol^{-1})
wild-type ABP[a]					
L-arabinose	24.8	2.4	0.096	0.098	−9.4
D-galactose	11.4	2.4	0.21	0.23	−8.9
D-fucose	13.9	43	3.09	3.8	−7.2
Met[108]Leu ABP					
L-arabinose	18.9	19.7	1.04	0.44 ± 0.02	−8.5
D-galactose	11.1	0.288	0.026	0.010[b]	−10.7
D-fucose	14.9	7.68	0.52	0.35 ± 0.04	−8.6

[a] For wild-type ABP, kinetic results are from Vermersch et al. (1990) and equilibrium results are from Miller et al. (1983). [b] The K_d for D-galactose binding by Met[108]Leu ABP was obtained from a single equilibrium dialysis competition experiment.

Table II: Data Collection and Refinement Statistics for the Structures of Met[108]Leu ABP Complexes Bound with D-Galactose, D-Fucose, and L-Arabinose

	D-galactose	D-fucose	L-arabinose	(target σ)
	Data Collection Statistics			
no. of obsd reflections	118025	120410	121531	
no. of unique reflections	45315	33726	35212	
R_{sym}	3.73	5.95	4.17	
resolution (Å)	8.00–1.49	8.00–1.67	8.00–1.67	
completeness (%)	88	91	95	
	Refinement Statistics			
no. of reflections used[a]	44720	29509	31106	
final R-factor[b]	0.175	0.162	0.160	
av $\|\|F_o\| - \|F_c\|\|$	25.28	35.86	34.52	
rms deviation in				
bond distances (Å)	0.020	0.022	0.024	0.020
angle distances (Å)	0.036	0.044	0.044	0.030
planar 1–4 distances (Å)	0.055	0.067	0.066	0.050
planarity (Å)	0.018	0.020	0.021	0.020
chiral volume (Å^3)	0.054	0.053	0.059	0.050
ω-angle (deg)	3.5	3.4	3.6	3.0
thermal parameters				
main-chain bond	1.534	1.692	1.781	1.500
main-chain angle	2.284	2.347	2.496	2.000
side-chain bond	4.069	3.991	4.082	2.500
side-chain angle	6.273	5.880	5.948	3.000
no. of nonhydrogen atoms	2547	2531	2541	
protein	2316	2316	2316	
sugar (α and β anomers)	24	22	20	
solvent	207	193	205	

[a] An F_o cutoff value of 2σ was used. [b] R-factor = $\sum||F_o| - |F_c||/\sum|F_o|$.

Kinetic Experiments. Ligand binding by ABP is rapid, reversible, and monophasic. The overall rate constants for the second-order binding of the different sugars to wild-type ABP have been previously determined (Miller et al., 1983; Vermersch et al., 1990). Binding rates (k_{obs}) were measured for Met[108]Leu ABP over a range of sugar concentrations. The overall second-order association rate constant was obtained as the slope of a plot of k_{obs} vs [sugar]. The overall association and dissociation rate constants (k' and k, respectively) for the binding of arabinose, fucose, and galactose to the Met[108]Leu ABP are listed in Table I. Relative to wild-type ABP, little change in the association rate of any of the sugars was obtained by the mutation at position 108.

The dissociation rates measured for the Met[108]Leu ABP–Fuc and –Gal complexes were more than 5 times slower than those for the corresponding wild-type protein–sugar complexes. The dissociation rate for the mutant ABP–Ara complex, however, was about 8-fold faster than that for the wild-type complex. Thus, as in the case of wild-type ABP, the differences in the affinities of the Met[108]Leu ABP for the different sugars can be attributed to differences in dissociation rates. Relative to wild-type ABP, the Met to Leu mutation appears to have further stabilized the ABP–Fuc and ABP–Gal complexes but has destabilized the ABP–Ara complex.

X-ray Crystallographic Structures of the Complexes of Met[108]Leu ABP with Sugars. A comparison of the structures of the mutant ABP complexed with the three monosaccharide ligands and their wild-type counterparts might reveal interactions that account for its altered binding properties. We, therefore, determined and refined the crystal structures of the complexes of Met[108]Leu ABP with Gal at 1.49-Å, Fuc at 1.67-Å, and Ara at 1.67-Å resolution. Refinements of the three structures were straightforward and resulted in the statistics shown in Table II. The electron density map of the Met[108]Leu ABP–Gal complex in the region of the Met to Leu substitution is shown in Figure 2.

As further judged by the lengths and angles of hydrogen bonds associated with the sugar in the binding site of the Met[108]Leu ABP, the position of the ligand in the mutant protein is very similar to that of wild type (Quiocho & Vyas, 1984; Quiocho et al., 1989). Besides Met[108], the two functional groups in the wild-type protein that interact with the sugar and rearrange to confer sugar specificity are Asp[89] and Wat[310] (Quiocho et al., 1989). Neither of these residues have noticeably changed in the structures of the Met[108]Leu ABP, complexed with the three sugars, relative to their positions in the wild-type structures.

Remarkably, the Met[108]Leu mutation results in a leucine side chain at position 108, which is essentially isosteric with the methionine side chain in the native ABP–Gal and ABP–

Table III: Apolar Contacts of Residue 108 in Wild-Type ABP and Met[108]Leu ABP Complexed with Sugar

		contact distance (Å)					
		wild-type ABP			Met[108]Leu ABP		
atom[a]	contact[b]	Gal	Ara	Fuc	Gal	Ara	Fuc
108 CB	His[259] CE1			3.99	4.04	3.97	3.87
	Ile[113] CD1		4.04	3.94			
108 CG	Ile[113] CD1			3.83			
	sugar C6[c]				3.95		3.96
108 CE	Arg[151] CZ		4.02				
	Trp[16] CH2		3.88				
	Ile[113] CD1	3.98					
	Ile[231] CG2	3.98					
	Pro[254] CB	3.51		3.63			
	Pro[254] CG	3.23		3.52			
	sugar C5[c]		4.02				
	sugar C6[c]	3.85		3.73			
108 CD1	Ala[110] CA				3.79	3.63	3.79
108 CD2	Pro[254] CB				3.96		3.84
	Pro[254] CG				3.84	3.99	3.82
	sugar C6[c]				3.72		3.73
total no. of contacts		5	4	6	6	3	6

[a] In the Met[108]Leu ABP 108 atoms, CB, CG, CD1, and CD2 correspond to leucine. In the wild-type ABP 108 atoms, CB, CG, and CE correspond to methionine. [b] Contacts are within a range of 4 Å. [c] Distances represent an average of those from α- and β-anomeric forms of the sugar.

Fuc complexes (Figures 2 and 3). The CD2 methyl group of Leu closely superimposes with the CE methyl group of Met. The apolar van der Waals contacts involving Leu[108] in the mutant ABP–sugar structures and those involving Met[108] in wild-type ABP–sugar structures are listed in Table III. These contacts are similar for the mutant and wild-type ABPs when complexed with Fuc and Gal. In the mutant ABP–Fuc and –Gal complexes, Leu[108] (like Met[108] in the wild-type structures) makes favorable contact with C6 of Fuc and Gal. Relative to its position in the complexes with Fuc and Gal, when Ara is bound, the Leu[108] side chain extends CD2 toward the space otherwise occupied by C6. This repositioning is similar to that which occurs with the Met[108] side chain in the wild-type ABP–Ara structure; however, because the Leu side chain is shorter, contact with C5 is not made. The lost apolar contact between Met[108] CE and C5 in the ABP–Ara complex could account for the decrease in affinity for Ara exhibited by the Met[108]Leu ABP relative to the wild type. The increase in affinity of the Met[108]Leu ABP for Gal and Fuc, however, cannot be explained by a change in the number of apolar contacts produced by the mutation.

The presence of an electronegative polar sulfur atom (SD) in the methionine side chain is an important difference between it and the leucine side chain. The possibility exists that the enhanced binding in the mutant ABP–Gal and –Fuc complexes is due to unfavorable interactions of the electronegative polar sulfur in the wild-type complexes that have been alleviated by the mutation. The sulfur atom makes three unfavorable contacts with side-chain carbons of Arg[151] and Ile[113] when Gal is bound (3.77–4.01 Å) and two when Fuc is bound (3.92 and 4.04 Å). In the ABP–Ara complex, Met[108] SD makes one unfavorable contact (with Ile[113] CD1 at 3.95 Å). Moreover, the Met[108] SD is positioned quite close to sugar C6 in the ABP–Gal (4.2 Å) and ABP–Fuc (4.38 Å) structures. The unfavorable proximity of the sulfur to the sugar is not present in ABP–Ara because Ara lacks C6 (the distance between SD and the closest Ara carbon, C5, is 5.63 Å) and because of the altered conformation of the Met[108] side chain in the complex with Ara relative to those with Fuc and Gal. Presumably, elimination of these unfavorable contacts by the Met to Leu mutation enhanced the affinity of Gal and Fuc by about 1.8 and 1.4 kcal mol^{-1}, respectively.

The Met[108]Leu ABP represents the second instance where the affinity of ABP has been increased and its specificity altered by site-directed mutagenesis. The substitution of Gly for Pro in the "hinge" region between the two domains of ABP increased the affinity for galactose by about 20-fold, while that for arabinose and fucose remained relatively unchanged (Vermersch et al., 1990). Interestingly, Met[108] was also postulated to have a role in the tighter binding of galactose exhibited by the Pro[254]Gly ABP. There is evidence that Met[108] is induced to swing away from C6 of the sugar and toward Ile[113] in the hinge by the binding of Gal (but not Ara and Fuc). Due to the resulting altered conformation of Ile[113], favorable apolar contacts are formed across the strands of the hinge in the Pro[254]Gly ABP–Gal relative to wild type. The higher affinity of this protein for galactose has been attributed to these new apolar contacts. In light of the results presented here, the galactose-bound form of the Pro[254]Gly ABP, like the Met[108]Leu ABP, may be additionally stabilized by the elimination of the unfavorable contact between the Met[108] sulfur atom and the sugar C6.

While hydrogen-bonding interactions predominate, van der Waals contacts significantly contribute to the stability of protein–carbohydrate interactions (Quiocho, 1986, 1989; Johnson et al., 1988). The extent of the nonpolar van der Waals contribution depends, in part, on the accessibility of nonpolar groups on the faces of the sugar. For example, L-arabinose bound to ABP has an exposed apolar patch consisting of C3H, C4H, and C5H, located on the B-face, which is sufficiently large to stack against the side chain of Trp[16] (Quiocho & Vyas, 1984; Vyas et al., 1988). This stacking interaction is a recurring molecular strategy found in protein–sugar interactions to increase affinity and confer specificity (Vyas et al., 1988; Johnson et al., 1988; Quiocho, 1986, 1989; Spurlino et al., 1991). The long flexible methionine side chain, with its terminal ε-methyl group, is well suited for contacting small nonpolar regions on the sugar face. The nonpolar portion of the A-face of L-arabinose consists primarily of the C2H group. Although this region is small and inaccessible to van der Waals contact by aromatic or branched hydrophobic residues, it contacts CE extended by Met[204] (Quiocho & Vyas, 1984; Vyas et al., 1988). With its flexibility to interact with C5 of Ara, and C6 of Fuc or Gal, the side chain of Met[108] fulfills the same function as that of Met[204] in stabilizing the protein–sugar complex through apolar–apolar contacts with CE. Additionally, our studies on the Met[108]Leu ABP have revealed an unusual negative role for Met[108] in conferring specificity via its ligand-dependent destabilizing, polar–apolar contacts with SD. Apparently this negative role can be counteracted by substitution with leucine.

Acknowledgments

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Registry No. Met, 63-68-3; Leu, 61-90-5; Ara, 5328-37-0; Gal, 59-23-4; Fuc, 3615-37-0.

References

Clark, A. F., Gerken, T. A., & Hogg, R. W. (1982) *Biochemistry 21*, 2227–2233.

Finzel, B. C. (1987) *J. Appl. Crystallogr. 20*, 53–55.

Hendrickson, W. A. (1985) *Methods Enzymol. 115*, 252–270.

Hogg, R. W., & Englesberg, E. (1969) *J. Bacteriol. 100*, 423-432.
Horazdovsky, B. F., & Hogg, R. W. (1987) *J. Mol. Biol. 197*, 27-35.
Johnson, L. N., Cheetham, J., McLaughlin, P. J., Acharya, K. R., Barford, D., & Phillips, D. C. (1988) *Curr. Top. Microbiol. Immunol. 139*, 81-134.
Kunkel, T. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488-492.
Miller, D. M., III, Olson, J. S., Pflugrath, J. W., & Quiocho, F. A. (1983) *J. Biol. Chem. 258*, 13665-13672.
Newcomer, M. E., Miller, D. M., III, & Quiocho, F. A. (1979) *J. Biol. Chem. 254*, 7529-7533.
Quiocho, F. A. (1986) *Annu. Rev. Biochem. 55*, 287-315.
Quiocho, F. A. (1989) *Pure Appl. Chem. 61*, 1293-1306.
Quiocho, F. A., & Vyas, N. K. (1984) *Nature 310*, 381-386.
Quiocho, F. A., Wilson, D. K., & Vyas N. K. (1989) *Nature 340*, 404-407.
Sack, J. (1988) *J. Mol. Graphics 6*, 244-245.
Schleif, R. (1969) *J. Mol. Biol. 46*, 185-196.
Scripture, J. T., Voelker, C., Miller, S., O'Donnell, R. T., Polgar, L., Rade, J., Horazdovsky, B. F., & Hogg, R. W. (1987) *J. Mol. Biol. 197*, 37-46.
Spurlino, J. C., Lu, G.-Y., & Quiocho, F. A. (1991) *J. Biol. Chem. 266*, 5202-5219.
Vermersch, P. S., Tesmer, J. J. G., Lemon, D. D., & Quiocho, F. A. (1990) *J. Biol. Chem. 265*, 16592-16603.
Vyas, N. K., Vyas, M. N., & Quiocho, F. A. (1988) *Science 242*, 1290-1295.

Removal of Salt from a Salt-Induced Protein Crystal without Cross-Linking. Preliminary Examination of "Desalted" Crystals of Phosphoglucomutase by X-ray Crystallography at Low Temperature†

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ABSTRACT: A model procedure for removing salt from relatively fragile salt-induced protein crystals is proposed. The procedure is based on physical principles and is validated by using millimeter-size crystals of rabbit muscle phosphoglucomutase grown from a 2.1 M solution of ammonium sulfate. Three types of operations are included in the procedure: initial transfer to salt solutions of reduced concentration; transfer to the organic-rich phase of an equilibrium biphasic mixture obtained with aqueous solutions of polyoxyethylene and the salt; and addition of various replacement cosolutes in aqueous solutions of polyoxyethylene to reduce osmotic stress on the crystal as the remaining salt is removed. A critical feature of the overall procedure is maintenance of near equilibrium throughout by using a large number of steps involving small changes in solute concentration. The conditions used in the actual transfer were adjusted to eliminate the fracturing of crystals by visually distinguishing between two opposing types of fracture patterns: those produced by osmotic crushing as opposed to osmotic expansion. Basic requirements for a successful procedure with other protein crystals are a high permeability toward small solutes and a relatively slow dissolution rate at salt concentrations for which biphasic mixtures can be obtained. Desalted crystals of phosphoglucomutase have no visible fractures, are stable in the final solution for at least a week, and exhibit no noticeable change in the resolution of their X-ray diffraction pattern. In fact, desalted crystals can be rapidly cooled to 160 K, whereas untreated crystals are almost completely disordered by the same cooling procedure. The component of the desalting mixture whose presence is crucial to the success of the cooling process is polyoxyethylene, which apparently impedes the formation of ice within the protein crystal. Diffraction data obtained with an area-detector diffractometer did not differ significantly, either in terms of quality or resolution range, between crystals in 2.3 M ammonium sulfate at room temperature and crystals at 160 K in which ammonium sulfate had been replaced by glycine. The successful use of the following replacement solutes, instead of glycine, also is documented: sucrose, glycerol, and a low molecular weight poly(ethylene glycol) (PEG-400).

In tetragonal crystals of rabbit muscle phosphoglucomutase grown from 2.1 M $(NH_4)_2SO_4$ (space group $P4_12_12$; a = 174 Å; c = 101 Å), the asymmetric unit is a dimer (M_r = 122000) that packs into a helical array around the unique crystallographic 4_1 screw axis (Lin et al., 1986). The helical array surrounds a channel with a diameter of about 40 Å centered on the 4_1 axis. A second channel, similar in average cross section, but with a wider variation in diameter, is centered on the parallel crystallographic 2_1 screw axis. The liquid phase, which accounts for approximately 60% of the volume of the crystal, occupies these channels and provides access to the active site of each enzyme molecule from the crystal surface through the 4_1 channel (Dai et al., to be published). The accessibility of the active site is sufficient to allow evaluation

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