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# Thermodynamic Analysis of Inducer Binding to the Lactose Repressor Protein: Contributions of Galactosyl Hydroxyl Groups and β Substituents†

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ABSTRACT: Kinetic and equilibrium studies of the binding of modified β-D-galactoside sugars to the *lac* repressor were carried out to generate thermodynamic data for protein–inducer interactions. The energetic contributions of the galactosyl hydroxyl groups to binding were assessed by using a series of methyl deoxyfluoro-β-D-galactosides. The C-3 and C-6 hydroxyls contributed $\leq -2.3$ and $-1.7 \pm 0.3$ kcal/mol to the binding free energy change, respectively, whereas the C-4 hydroxyl provided only a nominal contribution ($-0.1 \pm 0.2$ kcal/mol). Favorable contributions to the total binding free energy change were observed for replacement of *O*-methyl by *S*-methyl at the β-anomeric position and for *S*-methyl by *S*-isopropyl. Negative $\Delta H°$ values characteristic of protein–sugar complexes [Quiocho, F. A. (1986) *Annu. Rev. Biochem. 55*, 287–315] were observed for a series of β-D-galactosides differing at the β-glycosidic position. A decrease in $\Delta H°$ of ~6 kcal/mol upon replacement of the *O*-methyl substituent by *S*-methyl indicates a substantial increase in van der Waals' interactions and/or hydrogen bonding in this region of the ligand binding site. The more favorable free energy change for the binding of the *S*-isopropyl vs *S*-methyl compound is due mainly to more positive entropic contributions, consistent with an increase in apolar interactions. Thermodynamic parameters for isopropyl β-D-thiogalactoside (IPTG) binding at neutral pH are in agreement with previously published results [Butler, A. P., Revzin, A., & von Hippel, P. H. (1977) *Biochemistry 16*, 4757–4768; Donner, J., Caruthers, M. H., & Gill, S. J. (1982) *J. Biol. Chem. 257*, 14826–14829]. Arrhenius plots of kinetic rate constants for the binding of IPTG, methyl β-D-galactoside, and methyl β-D-thiogalactoside to the repressor revealed a protein structural transition at 12 °C. All of the experimental data are consistent with the hypothetical sugar binding site for repressor protein proposed by Sams et al. (1984) [Sams, C. F., Vyas, N. K., Quiocho, F. A., & Matthews, K. S. (1984) *Nature (London) 310*, 429–430].

Most effectors of the *lac* operon, either inducers or antiinducers, are β-D-galactosides (Monod et al., 1951; Müller-Hill et al., 1964). The in vivo inducer allolactose (1,6-*O*-β-D-galactopyranosyl-D-glucose) has been identified (Jobe & Bourgeois, 1972), and equilibrium association constants for the binding of an extensive series of β-D-galactoside derivatives to the *lac* repressor protein (Table I) have been determined (Barkley et al., 1975). Kinetic and equilibrium parameters for isopropyl β-D-thiogalactoside binding to *lac* repressor have been examined by fluorescence, visible and ultraviolet spectroscopy, and equilibrium dialysis (Friedman et al., 1976, 1977); the similarity in values obtained by these different methods confirms that the spectroscopic changes are proportional to the fractional saturation by ligand. However, the structural features of the carbohydrate ligand which determine its role as inducer or antiinducer remain elusive, and the specific amino acid residues contacting the ligand are unknown. Site-directed affinity labeling of the carbohydrate binding site with a series of analogues of D-glucose and D-galactose bearing reactive *N*-bromoacetyl functions at C-1 and C-2 of the pyranose ring was notably unsuccessful, although the absence of reactive amino acid residues is consistent with the repressor's role as a binding protein as opposed to a catalytic protein (Brown, 1979). In the absence of a definitive X-ray crystallographic description of free or liganded repressor protein, alternate methods must be relied upon to generate information about the nature of the protein–ligand interactions.

The availability of a series of methyldeoxyfluoro-substituted β-D-galactoside derivatives provided the opportunity to determine directly the contribution of the sugar hydroxyl groups to binding with repressor (Figure 1A). A hypothetical ligand binding site for lactose repressor (Sams et al., 1984; Figure 1B) has been derived from the solved X-ray crystallographic structure of ligand-bound L-arabinose binding protein (ABP)[1] (Quiocho & Vyas, 1984) and sequence homology between ABP and lactose repressor protein (Müller-Hill, 1983). The model indicates that the hydroxyl groups on C-2, -3, -4, and possibly -6 act as hydrogen-bond donors and acceptors to

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[1] Abbreviations: ONPF, *o*-nitrophenyl β-D-fucoside; IPTG, isopropyl β-D-thiogalactoside; ABP, arabinose binding protein; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Gal, galactoside.

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Table I: Effecting Ligands for *Lac* Repressor[a]

| ligand | $K_d$ (M) |
|---|---|
| inducers | |
| methyl β-D-galactoside | $1.0 \times 10^{-4}$ |
| methyl 1-thio-β-D-galactoside | $9.1 \times 10^{-6}$ |
| *n*-propyl 1-thio-β-D-galactoside | $7.7 \times 10^{-7}$ |
| isopropyl β-D-galactoside | $1.0 \times 10^{-4}$ |
| isopropyl 1-thio-β-D-galactoside | $8.3 \times 10^{-7}$ |
| *n*-butyl β-D-galactoside | $9.1 \times 10^{-6}$ |
| *n*-butyl 1-thio-β-D-galactoside | $5.0 \times 10^{-6}$ |
| benzyl β-D-galactoside | $1.0 \times 10^{-3}$ |
| benzyl 1-thio-β-D-galactoside | $4.3 \times 10^{-4}$ |
| 2-phenylethyl β-D-galactoside | $2.0 \times 10^{-4}$ |
| 2-phenylethyl 1-thio-β-D-galactoside | $1.3 \times 10^{-3}$ |
| *p*-aminophenyl β-D-galactoside | $1.9 \times 10^{-3}$ |
| *p*-aminophenyl 1-thio-β-D-galactoside | $1.3 \times 10^{-3}$ |
| *o*-nitrophenyl 1-thio-β-D-galactoside | $2.8 \times 10^{-3}$ |
| *p*-nitrophenyl β-D-galactoside | $2.3 \times 10^{-3}$ |
| *p*-nitrophenyl 1-thio-β-D-galactoside | $4.2 \times 10^{-2}$ |
| D-fucose | $4.5 \times 10^{-3}$ |
| galactose | $1.0 \times 10^{-3}$ |
| melibiose (6-*O*-α-D-galactopyranosyl-D-glucose) | $1.0 \times 10^{-4}$ |
| allolactose (6-*O*-β-D-galactopyranosyl-D-glucose) | $5.9 \times 10^{-7}$ |
| thioallolactose [6(*S*)-β-D-galactopyranosyl-D-glucose] | $3.0 \times 10^{-6}$ |
| neutral | |
| *o*-nitrophenyl β-D-galactoside | $1.1 \times 10^{-4}$ |
| antiinducers | |
| phenyl β-D-galactoside | $9.1 \times 10^{-4}$ |
| phenyl 1-thio-β-D-galactoside | $4.3 \times 10^{-3}$ |
| *o*-nitrophenyl β-D-fucoside | $1.4 \times 10^{-4}$ |
| *o*-nitrophenyl 1-thio-β-D-fucoside | $6.7 \times 10^{-3}$ |
| glucose | $7.1 \times 10^{-2}$ |
| lactose (4-*O*-β-D-galactopyranosyl-D-glucose) | $3.3 \times 10^{-1}$ |

[a] Affinities of ligands for repressor are reported as equilibrium dissociation constants. Original data, taken from Barkley et al. (1975), were reported as equilibrium association constants.

stabilize the bound β-galactoside. The thermodynamic parameters for ABP are consistent with values obtained for protein–ligand interactions stabilized mainly by hydrogen bonds and van der Waals' interactions (Quiocho, 1986). The nature of the molecular interactions in ligand-bound ABP suggests the utility of a thermodynamic description of repressor–inducer binding. In this study, the energetic contributions of the C-3, -4, and -6 hydroxyl groups of the galactose moiety to the stability of the sugar–repressor complex were assessed directly by measuring the rate and equilibrium constants for the binding of a series of fluoro-substituted β-D-galactosides. The contributions of the β-substituent were also determined by using a series of inducer sugars differing at the anomeric carbon atom.

## Materials and Methods

*Effectors.* Methyl 3-deoxy-3-fluoro-β-D-galactopyranoside, methyl 4-deoxy-4-fluoro-β-D-galactopyranoside, and methyl 6-deoxy-6-fluoro-β-D-galactopyranoside were obtained from Dr. C. P. J. Glaudemans and Dr. Paul Kovâc, Department of Health and Human Services, Section on Carbohydrates, NIH. Galactose, methyl β-D-galactoside, methyl β-D-thiogalactoside, ONPF, and IPTG were purchased from Sigma Chemical Co. Dilutions were prepared in TMS buffer (0.01 M Tris-HCl, pH 7.4, 0.001 M EDTA, 0.01 M $MgCl_2$, $1 \times 10^{-4}$ M DTT, and 0.2 M KCl).

*Buffers.* Temperature-dependent experiments were conducted in TMS buffer adjusted to pH 7.4 at each experimental temperature or in HMS buffer (0.01 M Hepes, 0.001 M EDTA, 0.01 M $MgCl_2$, $1 \times 10^{-4}$ M DTT, and 0.2 M KCl) adjusted to pH 7.4 at 20 °C. Identical results were obtained with either buffer, and most experiments employed the HMS buffer. Hepes reagent was obtained from Research Organics, Inc., and Sigma Chemical Co.

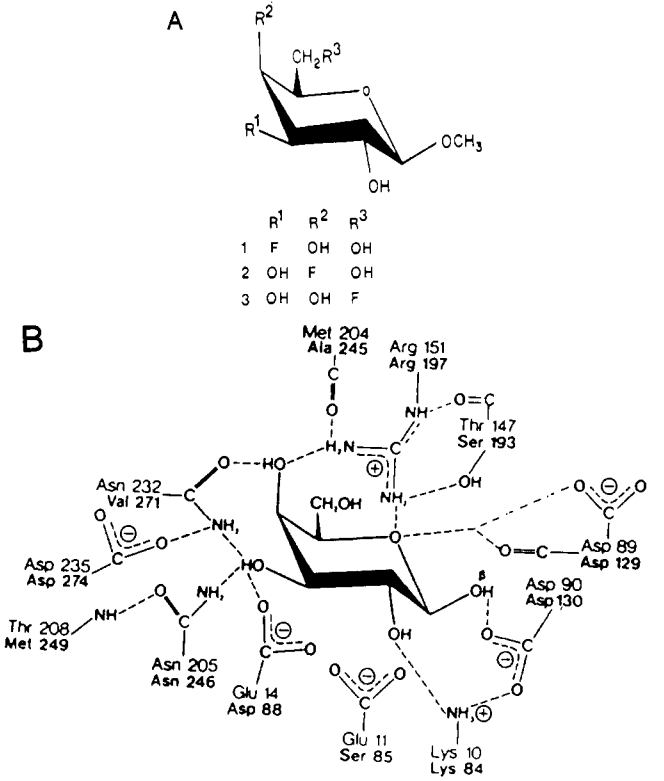


FIGURE 1: (A) Methyl β-D-galactoside derivatives deoxyfluoro-substituted at C-3, C-4, or C-6. (B) Amino acids in the ligand binding site for arabinose binding protein with homologous *lac* repressor amino acid substitutions indicated below by bold lettering. The nonanomeric hydroxyl groups in ABP are fully hydrogen bonded, acting as both donors and acceptors of hydrogen bonds (Sams et al., 1984).

*Repressor Purification and Characterization.* Lactose repressor protein was isolated from CSH46 cells, or from DH9 cells (HB101/$i^-$) containing pIQ plasmid (gifts from Dr. J. L. Betz) as described previously (O'Gorman et al., 1980). The purity was judged to be >95% as assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Operator–DNA and nonspecific DNA binding activity assays indicated that the repressor proteins were 80% active. The ultraviolet difference spectra and the fluorescence emission profiles, in the presence and absence of IPTG, were identical for both repressor preparations. Fluorometric titration by IPTG of each repressor preparation under identical conditions yielded similar results. Prior to each experiment, repressor was dialyzed overnight at 5 °C in the experimental buffer. The protein concentration was determined by the Bio-Rad protein assay (Bradford, 1976) with *lac* repressor of known concentration (by absorbance and amino acid analysis) as standard, and dilutions were made accordingly with the appropriate buffer.

*Fluorescence Equilibrium Measurements.* Fluorescence titration of repressor with effector was conducted on an SLM Series 400 polarization spectrofluorometer using an excitation wavelength of 285 nm (8-nm band-pass) and monitoring emission at wavelengths greater than 350 nm with a Corning 0-52 filter. Repressor protein (1.0 mL, $1.5 \times 10^{-7}$ M monomer) was placed in a cuvette and positioned in the thermostatic cuvette chamber. Following temperature equilibration, the initial fluorescence reading was recorded. At timed intervals of 1 min to allow equilibrium binding to be attained, a 2-μL aliquot of an effector stock solution was added to the cuvette; after each addition, the fluorescence was recorded. Additions were continued until saturation was achieved. The decrease in fluorescence at saturation was similar for all sugars: 20–30% of total fluorescence.

Table II: Equilibrium and Kinetic Rate Constants for Methyldeoxyfluoro-Substituted Sugars

| effector | $k_{assoc}$[a] ($M^{-1}$ $s^{-1}$ × $10^{-5}$) | $k_{dissoc}$[b] ($s^{-1}$) | $K_d$ (M) calcd $k_{dissoc}/k_{assoc}$ (×$10^5$) | $K_d$ (M) measured[c] (×$10^5$) |
|---|---|---|---|---|
| IPTG | 1.52 (±0.02) | 0.13 (±0.05) | 0.09 | 0.08 |
| Me β-D-thio-Gal | 2.03 (±0.1) | 3.7 (±1) | 1.82 (±0.5) | 2.4 |
| Me β-D-Gal | 0.64 (±0.05) | 13 (±4) | 20 (±7) | 15 |
| Me 6-deoxy-6-F-β-D-Gal | 0.24 (±0.09) | 84 (±24) | 350 (±160) | N.D.[d] |
| Me 4-deoxy-4-F-β-D-Gal | 0.95 (±0.14) | 24 (±5) | 25 (±6) | >10 |
| Me 3-deoxy-3-F-β-D-Gal | N.D.[d] | N.D.[d] | >1000 | N.D.[d] |
| galactose | 0.16 | 24 | 150 | 160 |

[a] Values were obtained by stopped-flow experiments at 20 °C. [b] Values were calculated from the intercept of the plot of $k_{obsd}$ vs effector concentration. [c] Values were measured by fluorescence titration. [d] N.D., not determined.

*Kinetic Measurements.* Kinetic parameters for inducer binding were measured with a Gibson-Dionex rapid-mixing stopped-flow spectrometer equipped with fluorescence optics. The excitation wavelength was 285 nm, and fluorescence emission was monitored by using a Corning filter to transmit wavelengths greater than 350 nm. Time courses were measured for the association of repressor with effector under pseudo-first-order conditions. For each ligand concentration, 4–12 traces were averaged and stored in the computer for further analysis. For temperature-dependent studies, the reactants were allowed to equilibrate for a minimum of 10 min at each experimental temperature in the thermostated chamber.

The IPTG dissociation rate constant for 20 °C was obtained by two methods. Binding of IPTG by repressor causes a characteristic decrease in total fluorescence at wavelengths greater than 350 nm; this shift is not observed for binding of the antiinducer ONPF. Therefore, IPTG displacement from repressor by rapid dilution into an equal volume of solution containing excess ONPF results in an increase in fluorescence as the antiinducer successfully competes for the sugar binding site. At very high ONPF and low IPTG concentrations, the rate-limiting step for this displacement is inducer dissociation from the protein. Dissociation rate constants for IPTG and several other inducers were also obtained by measuring the limiting association rates at low inducer concentrations, according to $k_{obsd} = k_{assoc}[\text{inducer}] + k_{dissoc}$. However, in all cases, direct determination of the dissociation rate constant is technically very difficult. ONPF absorbs strongly in the UV wavelength region and diminishes greatly the amount of exciting light which reaches the center of the stopped-flow cuvette. At the high ONPF concentrations required for displacement of IPTG, the resultant fluorescence change is quite small. At low or high temperatures, the real time course is easily obscured by small temperature gradients between the cuvette and the reactant solutions which produce spurious fluorescence changes. The extrapolation procedure using low inducer concentrations also requires the accurate measurement of small changes and is difficult at high and low temperatures. Thus, at most temperatures, $k_{dissoc}$ was calculated from the observed equilibrium constant and $k_{assoc}$. The values obtained by this method agreed with those measured by extrapolation where comparisons were possible (see Table II).

## Results and Discussion

*Methyldeoxyfluoro-Substituted and Other β-D-Galactosides.* Association of repressor protein with methyl 3-deoxy-3-fluoro-, methyl 4-deoxy-4-fluoro-, and methyl 6-deoxy-6-fluoro-β-D-galactoside was measured by stopped-flow rapid-mixing spectrometry. Methyl β-D-galactoside served as the standard for comparison to the methyldeoxyfluoro-substituted sugars, and galactose, methyl β-D-thiogalactoside, and IPTG were examined for further comparison. All time courses were fitted to a single-exponential expression, and the resultant parameters were used to determine the association rate constants from the slope of the linear plot of observed reaction rate vs inducer concentration. No curvature was observed at high sugar concentrations, consistent with previous measurements using IPTG (Friedman et al., 1976, 1977). The measured association rate constants for IPTG, methyl β-D-thiogalactoside, and methyl 4-deoxy-4-fluoro-β-D-galactoside were (1–2) × $10^5$ $M^{-1}$ $s^{-1}$; the association rate constants for galactose, methyl β-D-galactoside, and methyl 6-deoxy-6-fluoro-β-D-galactoside were 2–10-fold smaller, (1–6) × $10^4$ $M^{-1}$ $s^{-1}$ (Table II). The dissociation rate constants ranged from the directly measured value of 0.13 $s^{-1}$ for IPTG determined by ONPF displacement to the fitted value of ~90 $s^{-1}$ for methyl 6-deoxy-6-fluoro-β-D-galactoside. The binding of repressor to methyl 3-deoxy-3-fluoro-β-D-galactoside was not detectable in either kinetic or equilibrium experiments at concentrations up to 1 × $10^{-2}$ M.

Table III: Free Energy Changes for Substituted Sugars

| substitution | position | $\delta\Delta G°$ (kcal/mol)[a] source of $K_d$ values: kinetics[b] | equilibrium[c] |
|---|---|---|---|
| OH → F | C-4 | +0.1 (±0.2) | |
| OH → F | C-6 | +1.7 (±0.3) | |
| OH → F | C-3 | ≥+2.3 | |
| -*O*-Me → -*S*-Me | C-1, β | −1.4 (±0.3) | −1.1 |
| -*S*-Me → -*S*-isopropyl | C-1, β | −1.7 (±0.3) | −1.9 |
| -*O*-Me → galactose | C-1 | +1.2 (±0.6) | +1.4 |

[a] $\delta\Delta G° = \Delta G°_{substituted} - \Delta G°_{standard} = RT \ln (K_{d,substituted}/K_{d,standard})$ where the standard sugar is determined by the left-most substituent in column 1 (usually 1-*O*-methyl-β-D-galactoside). [b] The $K_d$ values were determined from the ratio of the kinetic rate constants at 20 °C as listed in Table II. [c] The $K_d$ values used were as measured in equilibrium titrations and are listed in Table II.

The equilibrium constant for each reaction was computed from the corresponding rate constants and was comparable to the value for the $K_d$ determined directly from fluorometric titration of repressor with effector (Table II). The Gibbs free energy, $\Delta G° = RT \ln K_d$, for binding of each fluoro-substituted sugar was calculated and compared to the corresponding value for *O*-methyl β-D-galactoside which was used as a standard:

$$\delta\Delta G° = \Delta G°_{substituted} - \Delta G°_{standard} = RT \ln (K_{d,substituted}/K_{d,standard}) \quad (1)$$

The resultant values for $\delta\Delta G°$ are shown in Table III. Substitution of a fluorine atom for a hydroxyl group at C-6 and C-3 results in significant loss of binding energy (≥2 kcal/mol). In contrast, substitution at C-4 causes a nominal loss of 0.1 ± 0.2 kcal/mol.

The contributions made by the hydroxyl groups at C-3, -4, and -6 were evaluated in terms of the sugar binding site of *lac* repressor proposed by Sams et al. (1984) (Figure 1B). Multiple hydrogen bonds are indicated at C-3 in the theoretical

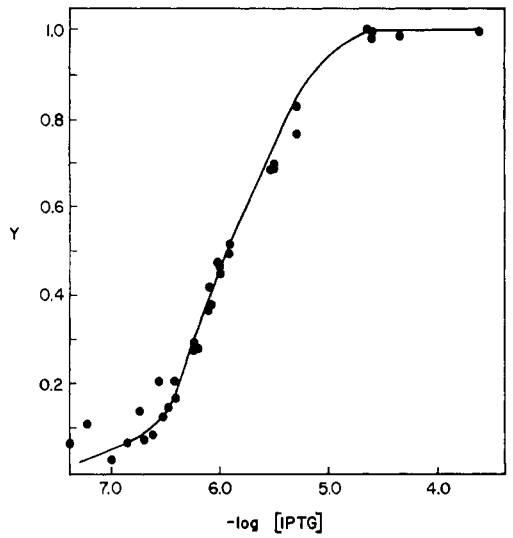


FIGURE 2: Saturation curve from the fluorometric titration of repressor protein by IPTG. Repressor protein at $1.5 \times 10^{-7}$ M in HMS buffer was equilibrated at the experimental temperature and titrated with IPTG as described under Materials and Methods. The fractional saturation ($Y$) is the observed decrease in fluorescence at a given inducer concentration relative to the total decrease in fluorescence. The $K_d$ is derived from the IPTG concentration at half-saturation by subtraction of half the repressor concentration.

site, which agrees with the experimental observation that the hydroxyl group at C-3 contributes $\leq -2.3$ kcal/mol to the overall binding free energy change. The bonding at C-6 is undefined in the arabinose binding protein due to the absence of a substituent in this position; however, comparison of rate and equilibrium constants determined for the binding of D-galactose and D-fucose to ABP indicated that the C-6 hydroxyl on galactose contributed $\sim -1.7$ kcal/mol to the binding free energy (Miller et al., 1983). In *lac* repressor, this hydroxyl also contributes $-1.7 \pm 0.3$ kcal/mol (Table III). At C-4, there is a hydrogen bond present in ABP between Asn-232 and the sugar hydroxyl. In *lac* repressor, Val-271 is thought to be substituted for the asparagine residue. Since the isopropyl side chain of valine cannot participate in hydrogen bonding, substitution of a fluorine atom at C-4 is predicted to have little effect in the *lac* repressor as was observed (Table III). Unusual bidentate H bonds are formed in ABP between Asn-232 and the C-3 and C-4 hydroxyl groups of arabinose (Quiocho & Vyas, 1984); the presence of Val-271 in the *lac* repressor, while consistent with results for the C-4 fluoro sugar, would dictate an alternative bonding arrangement to C-3. Similarly, in ABP, one portion of the guanidino group in Arg-151 is thought to hydrogen bond to the hydroxyl at C-4; evidently, this interaction is not important in the *lac* repressor.

Results from the stopped-flow experiments using inducer molecules which differ at the $\beta$ linkage indicated that addition of the 1-methyl group to galactose contributed $-1.2 \pm 0.6$ kcal/mol to the binding free energy change and replacement of the oxygen atom of methyl $\beta$-D-galactoside by a sulfur atom to give methyl $\beta$-D-thiogalactoside yielded a more favorable $\Delta G°$ by $-1.4 \pm 0.3$ kcal/mol. Replacement of the methyl group by an isopropyl group at the sulfur atom also resulted in a more favorable $\Delta G°$ by $-1.7 \pm 0.3$ kcal/mol.

*Temperature Dependence Studies. (A) Equilibrium Enthalpy Changes.* Fluorometric titrations of repressor protein with inducer were conducted at various temperatures from 5 to 35 °C. The data, corrected for background by a control titrated with buffer additions, were plotted as fractional saturation ($Y$) vs log [inducer]; the $K_d$ was determined from half-saturation of the binding curve (Figure 2). van't Hoff

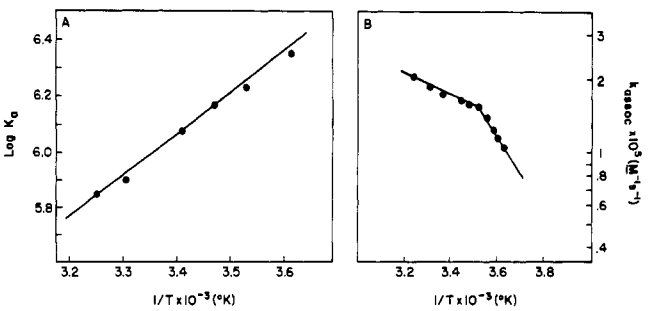


FIGURE 3: van't Hoff and Arrhenius plots for IPTG binding to repressor. (A) Equilibrium dissociation constants were obtained from IPTG titration curves as described in Figure 3 at various temperatures. Enthalpy was calculated from the slope of the graph according to the relation slope $= -\Delta H°/R$. The slope has a coefficient of determination of 0.99. (B) Arrhenius plot of the association rate constants for the binding of repressor protein to IPTG. The energies of activation (slope $= -E_a/R$) for association both above and below the break were determined (Table V).

Table IV: Temperature-Dependent Thermodynamic Parameters for Inducer Binding to Repressor Protein

| sugar | temp (°C) | $\Delta H°$ [a] (kcal/mol) | $\Delta G°$ [b] (kcal/mol) ($\Delta G° = -RT \ln K_a$) | $\Delta S°$ [c] (cal K$^{-1}$ mol$^{-1}$) [$\Delta S° = (\Delta H° - \Delta G°)/T$] |
|---|---|---|---|---|
| galactose | 20 | −3.9 | −3.8 | −0.3 |
| Me $\beta$-D-Gal | 20 | −1.3 | −5.2 | 13.3 |
| Me $\beta$-D-thio-Gal | 20 | −7.1 | −6.3 | −2.7 |
| IPTG | 20 | −6.2 | −8.2 | 6.8 |

[a] Determined from the slope of the van't Hoff plot. Equilibrium dissociation constants were obtained from inducer titration curves at various temperatures. No discontinuities were observed. [b] Determined directly from $K_d$ values obtained by fluorescence titration equilibrium experiments. [c] Calculated from $\Delta H°$ and $\Delta G°$.

plots of the data were constructed to determine the equilibrium enthalpy change, $\Delta H°$ (Figure 3A).

A thermodynamic evaluation of binding to repressor was obtained for the inducers galactose, methyl $\beta$-D-galactoside, methyl $\beta$-D-thiogalactoside, and IPTG. The results (Table IV) show that these inducers have negative values for $\Delta H°$, which is characteristic of complexes stabilized mainly by hydrogen bonds and van der Waals' interactions (Ross & Subramanian, 1981) as well as protonation events coupled to binding, for example. The significantly large differences in $\Delta H°$ which are observed for the various $\beta$ substituents, in conjunction with concomitant changes in $\Delta S°$, reflect the interplay of contributions from polar and nonpolar components in the repressor–inducer complex, as other effects on $\Delta H°$ (e.g., protonation and conformational changes) would presumably be common to the binding of different inducers. The thermodynamic parameters for the binding of galactose were calculated by assuming that 64% of the sugar was in the $\beta$-pyranose configuration (Pigman & Horton, 1972). Replacement of the hydrogen atom at C-1 by a methyl group eliminates the potential for hydrogen bonding by the anomeric hydroxyl group as observed in ABP and introduces a hydrophobic group. The thermodynamic parameters for methyl $\beta$-D-galactoside reflect this change by more positive values for $\Delta H°$ and $\Delta S°$ as compared to galactose. A dramatic decrease of $\sim 6$ kcal/mol in $\Delta H°$ is seen for the binding of methyl $\beta$-D-thiogalactoside as compared to methyl $\beta$-D-galactoside. This large difference may indicate (1) a local conformational change resulting in an increase in van der Waals' interactions at multiple sites rather than simply a localized interaction at the sulfur atom, (2) changes in number, type, or directionality of H bonds, or

Table V: Temperature-Dependent Activation Parameters for Inducer Binding to Repressor

| sugar | temp (°C) | $E_{a_{assoc}}$ [a] (kcal/mol) | $A_{assoc}$ [b] ($M^{-1}$ $s^{-1}$) | $E_{a_{dissoc}}$ [c] (kcal/mol) | $A_{dissoc}$ [b] ($s^{-1}$) |
|---|---|---|---|---|---|
| galactose | 5 | 14.2 | $8.4 \times 10^{14}$ | 18.1 | $8.8 \times 10^{14}$ |
| | 20 | 8.4 | $3.0 \times 10^{10}$ | 12.3 | $3.6 \times 10^{10}$ |
| Me β-D-Gal | 5 | 13.6 ± 0.8 | $1.2 \times 10^{15}$ | 14.9 | $1.3 \times 10^{12}$ |
| | 20 | 7.6 ± 0.5 | $3.0 \times 10^{10}$ | 8.9 | $5.7 \times 10^{7}$ |
| Me β-D-thio-Gal | 5 | 7.7 ± 0.4 | $1.4 \times 10^{11}$ | 14.8 | $5.6 \times 10^{11}$ |
| | 20 | 3.4 ± 0.9 | $7.0 \times 10^{7}$ | 10.5 | $2.5 \times 10^{8}$ |
| IPTG | 5 | 5.7 ± 1.7 | $3.5 \times 10^{9}$ | 11.9 | $1.0 \times 10^{8}$ |
| | 20 | 2.2 ± 0.3 | $6.7 \times 10^{6}$ | 8.4 | $2.4 \times 10^{5}$ |

[a] Determined from the slope of the Arrhenius plot of the association rate constants obtained by stopped-flow experiments. [b] $A = k_{assoc}$ or $k_{dissoc}/e^{-E_a/RT}$. [c] The activation energy for dissociation was calculated from $E_{a_{dissoc}} = E_{a_{assoc}} - \Delta H°$. $\Delta H°$ was taken from Table IV. These values are not well-defined for galactose. The errors in $E_a$ for dissociation are roughly ±1.5–3 kcal/mol.

(3) alterations in solvent accessibility as a consequence of binding.

There is a positive contribution of ~1 kcal/mol to $\Delta H°$ and ~10 cal $K^{-1}$ $mol^{-1}$ to $\Delta S°$ upon replacement of the methyl group by an isopropyl group at the β-sulfur linkage. Positive contributions to $\Delta S°$ derived from hydrophobic associations (Ross & Subramanian, 1981) may be ascribed to the two additional methyl groups present on IPTG. The more positive $\Delta H°$, by 0.9 kcal/mol, suggests fewer van der Waals' interactions and/or H bonds. Steric hindrance by the isopropyl group could interfere with the van der Waals' interactions at the sulfur atom, or, alternatively, this bulky group could exclude water molecules which have been shown in ABP to play an essential role in mediating interactions between the sugar and amino acids at the active site (Quiocho, 1986). The van't Hoff enthalpy reported here (−6.2 kcal/mol) for IPTG binding is identical with the value determined previously by Butler et al. and is similar to the value (−5.9 kcal/mol) determined by Donner et al. (Butler et al., 1977; Donner et al., 1982). Our results support the conclusions made by Donner et al. that IPTG binding involves minimal hydrophobic interactions and likely is mediated by hydrogen bonding. These interpretations are also consistent with the conclusion by Butler et al. that IPTG binding is largely enthalpically driven. Our study reveals that a large contribution to $\Delta H°$ arises from van der Waals' interactions (or hydrogen bonding) between the protein and the sulfur atom at the C-1 position.

(*B*) *Activation Energy Parameters.* Association rate constants at temperatures from 3 to 36 °C were determined for the binding of galactose, methyl β-D-galactoside, methyl β-D-thiogalactoside, and IPTG to the *lac* repressor. Arrhenius plots of the association rate constants (Figures 3B and 4) revealed a discontinuity in the slope at 12 °C, as noted previously for repressor and IPTG binding (Whitson et al., 1986). The energies of activation, $E_{a_{assoc}}$, were determined from the slopes above and below the temperature discontinuity (Table V). The activation energies for IPTG at all temperatures are smaller than those for the other sugars; in addition, the ratio of $E_{a_{assoc}}$ at high and low temperatures is the largest (2.6-fold) for this inducer. It is apparent that the presence of the sulfur atom significantly lowers the activation energy, especially at temperatures >12 °C, and that the isopropyl group further enhances the diminution. The discontinuity seen for association of the sugars and repressor suggests that there is a conformational transition in the protein at 12 °C. In contrast, the equilibrium thermodynamic parameters remain constant over the temperature range 5–35 °C and do not reflect the apparent structural change at 12 °C (Figure 3A,B). Activation energy barriers for dissociation were computed from the equilibrium enthalpy change and $E_a$ for association. The smallest activation energy for dissociation (Table V) was observed for IPTG at all temperatures. The ratio of $E_{a_{dissoc}}$, above and below 12 °C, remains essentially constant for all sugars.

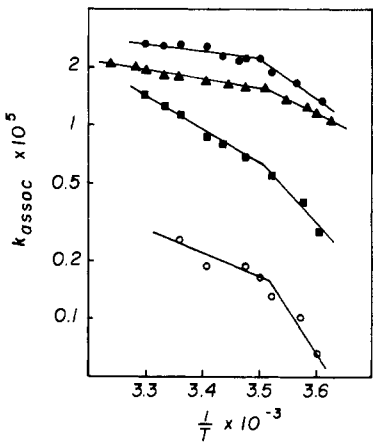


FIGURE 4: Arrhenius plots for the association of repressor with sugar ligands. (●) Methyl β-D-thiogalactoside; (▲) IPTG; (■) methyl β-D-galactoside; (○) galactose. Plots represent data from one series of temperature-dependent binding experiments. Values for $E_a$ (Table V) represent the average of three to five series of temperature-dependent experiments for each sugar.

Thus, the nature of the β substituent is not the limiting factor in surmounting the energy barrier for dissociation.

## CONCLUSIONS

Measurements of the binding of a series of methyldeoxyfluoro-substituted sugars to the *lac* repressor have demonstrated that the energetic contributions of the hydroxyl groups vary markedly with position on the galactose ring. The loss of a hydroxyl group at the C-3 position effectively eliminates binding of the ligand, whereas the loss of the C-4 hydroxyl appears to be inconsequential. The important role of the C-6 hydroxyl in binding has been confirmed by these experiments. Interpretation of our results is consistent with a hypothetical sugar binding site for the *lac* repressor (Sams et al., 1984). The postulated bonding at C-3, in which the hydroxyl group participates in three hydrogen bonds, is consistent with the magnitude of the loss of ≥2.3 kcal/mol to the binding energy for the fluoro sugar at C-3. The loss in binding energy for the fluoro sugar at C-6 (1.7 ± 0.3 kcal/mol) suggests that this hydroxyl also participates in multiple hydrogen bonds and is identical with the loss of 1.7 kcal/mol of free energy observed for the binding of D-fucose to ABP as compared to the binding of D-galactose. The minimal effects resulting from changes at C-4 imply no net loss of contacts at this position upon fluorosubstitution.

The negative values of $\Delta H°$ observed for the inducers are characteristic of complexes stabilized by H bonds and van der Waals' interactions and are consistent with other protein–sugar complexes, with the exception of hexokinase (Quiocho, 1986). The large negative contribution to $\Delta H°$, as a direct or indirect consequence of the presence of the sulfur atom at C-1, suggests that the resultant van der Waals' interactions and/or hydrogen

bonds parallel the effectiveness of induction by a sugar. The order of effectiveness of ligand induction is IPTG > methyl β-D-thiogalactoside > methyl β-D-galactoside > galactose (Müller-Hill et al., 1964; Riggs et al., 1970; Barkley et al., 1975).

Discontinuities in Arrhenius plots have been observed for some enzymes, e.g., fumarate hydratase in acid solution and myosin ATPase reaction with inosine triphosphate or adenosine triphosphate in the presence of dinitrophenol or actin (Dixon & Webb, 1964). Generally, these discontinuities have been ascribed to multiple forms of the enzyme in equilibrium with one another; these forms all exhibit activity but display different activation energies for the reaction. If the effect of temperature on the transition between the forms is significant, a discontinuity will be observed in the Arrhenius plots. The Arrhenius plots of rate constants for association of repressor with galactose, methyl β-D-galactoside, methyl β-D-thiogalactoside, and IPTG all reveal a temperature-dependent structural transition in repressor at 12 °C. The energy barrier to association with the repressor protein for methyl β-D-thiogalactoside and IPTG is lower than for galactose and the *O*-methyl sugar even below 12 °C; at temperatures above 12 °C, the energy of activation for IPTG is the lowest. Since discontinuities are not seen in the van't Hoff plots of the equilibrium constants for sugar binding, structural alteration in the protein affecting its kinetic characteristics appears to influence only the transition state of the binding process and not the initial and final states.

Marked changes have also been observed for the activation energy of repressor–operator DNA dissociation around 10–15 °C, and similar breaks were observed in van't Hoff plots of the DNA equilibrium binding constant (Whitson et al., 1986). However, discontinuities were not observed in Arrhenius plots of the temperature dependence of the DNA–repressor association rate constant. The rate-limiting steps for repressor–operator association are probably diffusion up to and binding with nonspecific DNA, since sliding along the DNA to the operator region is thought to be very rapid. Thus, the DNA association reaction is thought to be relatively insensitive to the protein structural changes including those elicited by sugar binding.

By analogy with the periplasmic binding proteins, the speed of inducer binding to the *lac* repressor is thought to be dependent on the rate and extent of opening of the sugar binding cleft (Miller et al., 1983). One possible interpretation of the temperature dependence of the sugar binding kinetics is that the rate of opening and closing of the active site ["hinge bending" as described by Mao et al. (1982) for ABP] is affected by temperature in a non-Arrhenius manner. Above 12 °C, this process may be too fast to influence directly the observed association and dissociation rate constants; however, below the transition temperature, it could become rate limiting.

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